



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:  
George H. Yoo

Serial No.: 10/747,798

Filed: December 29, 2003

For: p53 TREATMENT OF  
PAPILLOMAVIRUS AND  
CARCINOGEN-TRANSFORMED CELLS  
IN HYPERPLASTIC LESIONS

Group Art Unit: 1633


Examiner: Scott D. Priebe

Atty. Dkt. No.: INRP:104US

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BRIEF ON APPEAL

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## TABLE OF CONTENTS

I.	REAL PARTY IN INTEREST.....	2
II.	RELATED APPEALS AND INTERFERENCES .....	2
III.	STATUS OF THE CLAIMS .....	2
IV.	STATUS OF AMENDMENTS.....	2
V.	SUMMARY OF CLAIMED SUBJECT MATTER.....	2
VI.	GROUND OF REJECTION TO BE REVIEWED ON APPEAL .....	3
	1) Whether claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 are properly rejected under 35 U.S.C. §102(a) as being anticipated by Clayman, G. (Ref. C95 of the IDS filed August 16, 2004; "Clayman;" Exhibit 1), as evidenced by Oda <i>et al.</i> (Carcinogenesis 17(9):2003-2008, 1996; Exhibit 2) and Flaitz <i>et al.</i> (Oral Oncol. 34:448-453, 1998; Exhibit 3), and as evidenced by Recombinant DNA Advisory Committee (RAC) (Minutes of Meeting dated March 8, 2001, U.S. Dept. of Health and Human Services; "RAC Meeting Minutes," Exhibit 4) .....	3
VII.	ARGUMENT.....	4
A.	Rejection of Claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 Under 35 U.S.C. §102(a) as Being Anticipated by Clayman, as Evidenced by Oda <i>et al.</i> and Flaitz <i>et al.</i> , and as Evidenced by RAC Meeting Minutes.....	5
	1. Clayman Does Not Expressly or Inherently Disclose Papillomavirus-Transformed Cells.....	5
	2. Clayman Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, 33, and 54.....	7
	a) "a keratinocyte" .....	7
	b) "a skin cell" (claim 6).....	8
	c) a "douche solution" (claims 18, 33, and 54).....	8
	d) "a liquid carrier formulated for vaginal delivery" (claim 33) .....	8
	3. Conclusion.....	9
B.	Rejection of Claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 Under 35 U.S.C. §102(b) as Being Anticipated by RAC, as Evidenced by Oda <i>et al.</i> and Flaitz <i>et al.</i> .....	9
	1. RAC Does Not Expressly or Inherently Disclose Papillomavirus-Transformed Cells .....	9
	2. RAC Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, 33, and 54.....	10

3.	Conclusion.....	11
C.	Rejection of Claims 1-14, 19-29, 38-50, and 55-60 Under 35 U.S.C. §102(b) as Being Anticipated by Nielsen as evidenced by Oda <i>et al.</i> and Flaitz <i>et al.</i> ....	11
1.	Nielsen Does Not Expressly or Inherently Disclose HPV-Transformed Cells .....	11
2.	Nielsen Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, 33, and 54.....	11
3.	Conclusion.....	12
D.	Rejection of Claims 1-15, 18-29, 33, 38-51, and 54-60 Under 35 U.S.C. 102(b) as Being Anticipated by El-Deiry .....	12
1.	El-Deiry Does Not Anticipate Claims 1-15, 18-29, 38-51, and 54-60 Because it Does Not Expressly or Inherently Disclose Any Method that Involves Administration of a Polynucleotide Encoding a p53 Polypeptide to an Papillomavirus-Transformed Cell.....	12
2.	El-Deiry Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, and 54.....	14
3.	El-Deiry Does Not Expressly or Inherently Anticipate Claim 33 .....	14
4.	Conclusion.....	14
E.	Rejection of Claims 16, 17, 31, 32, 52, and 53 as Being Unpatentable Under 35 U.S.C. §103(a) Over Either RAC as Evidenced by Oda <i>et al.</i> and Flaitz <i>et al.</i> , or El-Deiry in View of Zhang <i>et al.</i> .....	14
VIII.	CONCLUSION .....	16

APPENDIX 1: Listing of Appealed Claims

APPENDIX 2: Evidence Appendix

APPENDIX 3: Related Proceedings Appendix



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**BRIEF ON APPEAL**

**Mail Stop Appeal Brief - Patents**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Appellant hereby resubmits this Appeal Brief in response to the Final Office Action dated April 14, 2006. The Notice of Appeal was inadvertently omitted when this Brief was filed on July 10, 2006. Therefore, this Brief is filed pursuant to the Notice of Appeal concurrently filed herewith. The final Office Action was mailed on April 14, 2006, therefore, no extension fees are believed to be due.

The fee for filing this Appeal Brief was paid with the Brief mailed on July 10, 2006. No additional fees are believed due in connection with this paper. However, should any other fees be due, or the enclosed fee be deficient or absent, the Commissioner is authorized to withdraw the appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/INRP:104US.



### **I. REAL PARTY IN INTEREST**

The real party in interest is the assignee, Introgen Therapeutics, Inc., Austin, Texas.

### **II. RELATED APPEALS AND INTERFERENCES**

There are no related appeals and interferences.

### **III. STATUS OF THE CLAIMS**

The application was filed with original claims 1-60. In response to the Office Action dated November 17, 2005, an Amendment was submitted in which claims 31, 52, and 53 were amended and claims 30 and 34-37 were canceled without prejudice or disclaimer. Thus, claims 1-29, 31-33, and 38-60 are currently pending in the application and are the subject of this appeal. Appendix 1 includes a listing of the currently pending claims, which are the claims that are the subject of this appeal.

### **IV. STATUS OF AMENDMENTS**

No amendments were filed subsequent to the final rejection.

### **V. SUMMARY OF CLAIMED SUBJECT MATTER**

The claimed subject matter concerns methods for inhibiting the growth of a papillomavirus-transformed cell in a hyperplastic lesion in a subject by topically administering to the subject a composition that includes (1) an expression cassette that includes a promoter, active in the cells of the lesion, operably linked to a polynucleotide encoding a p53 polypeptide, and (2) a pharmaceutical preparation suitable for topical delivery, wherein expression of the p53

polypeptide inhibits growth of the cell. Specification, page 4, lines 20-27. The claimed subject matter also concerns formulations for inhibiting the growth of a papillomavirus-transformed cell in a hyperplastic lesion in a subject that include an expression cassette that includes a promoter operably linked to a polynucleotide encoding a p53 polypeptide and a liquid carrier, such as formulations suitable for delivery as a mouthwash or a douche solution. Specification, page 6, lines 3-11. The claimed subject matter is also directed to methods of suppressing or preventing papillomavirus-mediated transformation of a cell in a subject that involves administering to the cell a composition that includes (1) an expression cassette that includes a promoter, active in the cell, operably linked to a polynucleotide encoding a p53 polypeptide, and (2) a pharmaceutical preparation suitable for topical delivery, wherein expression of p53 suppresses transformation. Specification, pages 17-22.

## **VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

1) Whether claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 are properly rejected under 35 U.S.C. §102(a) as being anticipated by Clayman, G. (Ref. C95 of the IDS filed August 16, 2004; "Clayman;" **Exhibit 1**), as evidenced by Oda *et al.* (Carcinogenesis 17(9):2003-2008, 1996; **Exhibit 2**) and Flaitz *et al.* (Oral Oncol. 34:448-453, 1998; **Exhibit 3**), and as evidenced by Recombinant DNA Advisory Committee (RAC) (Minutes of Meeting dated March 8, 2001, U.S. Dept. of Health and Human Services; "RAC Meeting Minutes," **Exhibit 4**).

2) Whether claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 are anticipated under 35 U.S.C. §102(b) by the RAC, as evidenced by Oda *et al.* and Flaitz *et al.*

3) Whether claims 1-14, 19-29, 38-50, and 55-60 are anticipated under 35 U.S.C. §102(b) by Nielsen *et al.* (U.S. Patent App. Pub. No. 2001/0044420; **Exhibit 5**), as evidenced by Oda *et al.* and Flaitz *et al.* with respect to claims 1-14, 19-29, 38-50, and 55-60.

4) Whether claims 1-15, 18-29, 33, 38-51, and 54-60 are anticipated by El-Deiry (WO 99/66946; **Exhibit 6**) under 35 U.S.C. §102(b).

5) Whether claims 16, 17, 31, 32, 52, and 53 are properly rejected under 35 U.S.C. §103(a) as being unpatentable over either (i) RAC Meeting Minutes, as evidenced by Oda *et al.* and Flaitz *et al.*, as applied to claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 above, or (ii) El-Deiry as applied to claims 1-15, 18-29, 33, 38-51, and 54-60 above, and further in view of Zhang *et al.* (WO 00/29024; **Exhibit 7**).

## VII. ARGUMENT

As an initial matter, Appellant notes that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by “substantial evidence” within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that “the ‘substantial evidence’ standard asks whether a reasonable fact finder could have arrived at the agency’s decision.” *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner’s position on Appeal must be supported by “substantial evidence” within the record in order to be upheld by the Board of Patent Appeals and Interferences.

**A. Rejection of Claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 Under 35 U.S.C. §102(a) as Being Anticipated by Clayman, as Evidenced by Oda *et al.* and Flaitz *et al.*, and as Evidenced by RAC Meeting Minutes**

**1. Clayman Does Not Expressly or Inherently Disclose Papillomavirus-Transformed Cells**

Clayman does not anticipate the claims because it fails to expressly or inherently describe each limitation of the claimed invention. See *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987) (“A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference.”). It is agreed by the Examiner that Clayman does not state or disclose that the premalignancies or cancers being treated include cells infected with HPV. See Office Action dated April 14, 2006. Thus, there is no express anticipation.

Nor is there inherent anticipation. The Examiner, citing Oda *et al.* and Flaitz *et al.*, argues that HPV infection of treated cells in Clayman is inherent because “one can be certain that in the population of individuals with oral or cervical cancer who are to be treated with the method of Clayman,” that “half or most will have HPV infected cells in the premalignancy or cancer.” Office Action dated April 14, 2006, page 4. However, neither Oda *et al.* nor Flaitz *et al.* nor any other documents of record support the Examiner’s assertion that “one can be certain” that half or most of the lesions set forth in Clayman contain HPV-infected cells. In fact, a substantial fraction of oral and cervical cancers are not infected with HPV. See, *e.g.*, Oda *et al.*, page 2003. The Examiner appears to be relying on facts within his own personal knowledge which, in accordance with 37 C.F.R. §1.104(d)(2) is impermissible because no specific data to support this rejection (such as an affidavit) have been set forth.

Further, the Examiner has misinterpreted the case law pertaining to inherent anticipation. Inherent anticipation arises when “the prior art necessarily functions in accordance with, or

includes, the claimed limitations.” *Atlas Powder Co.*, 190 F.3d at 1347. (citing *In re King*, 801 F.2d 1324, 11326 (Fed. Cir., 1986); see also *Atlas Powder Co.*, 190 F.3d at 1347-48). The Examiner has established, by citing Oda and Flaitz, that ***not every malignancy or premalignancy of the cervix or oral cavity involves cells that are infected with HPV.*** (emphasis added). Thus, he has established that it is ***not necessary*** for a malignancy or premalignancy of the cervix or oral cavity to include cells infected with HPV.

The present invention is not directed to obtaining patent protection of a method of inhibiting the growth of cells that are not infected with HPV. That the claimed method might be applied in the treatment of cells not infected by HPV is not relevant to the issue of whether Clayman anticipates the claimed invention. The issue is whether it would be inherent that every cancer set forth in Clayman would be infected with HPV. Clayman provides no explicit teaching. In fact, Oda and Flaitz support the notion that it is NOT inherent that every cancer set forth in Clayman would be infected with HPV.

It is well-established that:

“inherency ... ***may not be established by probabilities or possibilities.***  
The mere fact that a certain thing may result from a given set of circumstances is not sufficient.”

*MEHL/Biophile Int’l Corp. v. Milgraum*, 192 F.3d 1362, 1365 (Fed. Cir. 1999) (quoting *In re Oelrich*, 666 F.2d 578, 581 (CCPA 1981) (emphasis added). The Examiner argues that “***it may be probable*** that a premalignancy or cancer in one given individual would include HPV infected cells, the evidence presented in Oda and Flaitz show that one can be certain that in the population of individuals with oral or cervical cancer who are to be treated with the method of Clayman, RAC minutes, or Nielsen, half or most will have HPV infected cells in the premalignancy or cancer.” Office Action dated April 14, 2006 (emphasis added). In essence, he argues that the a

possibility that such a lesion might include HPV infected cells is sufficient to establish inherent anticipation. However, such an interpretation of inherent anticipation is not in line with established case law.

In *MEHL/Biophile*, Milgraum contended that all of the claims of MEHL/Biophile's patent directed to a method for removing hair using a laser were anticipated by an instruction manual. See *MEHL/Biophile*, 192 F.3d 1362. The claims included the step of "aligning a laser light applicator substantially vertically over a hair follicle opening." *Id.* The manual taught aiming the laser at skin pigmented with tattoo ink. Milgraum contended that the claims were inherently anticipated because an operator of the laser could use the laser according to the manual without necessarily aligning the laser substantially vertically over a hair follicle opening. *Id.* The Court held that "the possibility of such an alignment does not legally suffice to show anticipation" and that "[o]ccasional results are not inherent." *MEHL/Biophile*, 192 F.3d at 1365. Similarly, the mere possibility that one of the lesions set forth in Clayman might contain HPV DNA is not sufficient to establish inherent anticipation. As noted in *MEHL/Biophile*, *occasional results are not inherent*. Without an inherent teaching regarding HPV-transformed cells, there can be no anticipation.

**2. Clayman Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, 33, and 54**

**a) "a keratinocyte"**

Clayman does not expressly or inherently disclose the limitation "wherein said cell is a keratinocyte" in claim 4. Appellant identifies no disclosure in Clayman pertaining to any malignancy or premalignancy involving keratinocytes. The patients set forth in Clayman have preneoplastic lesions of the oral cavity. Most of the oral cavity is lined by nonkeratinized

squamous epithelium, and not keratinocytes. Therefore, Clayman does not expressly or inherently disclose any cell that is a keratinocyte.

**b) “a skin cell” (claim 6)**

Appellant points out that Clayman does not expressly or inherently disclose treatment of “a skin cell,” as set forth in claim 6. As set forth above, Clayman pertains to treatment of oral cavity, which is not lined by skin cells. Therefore, Clayman additionally fails to anticipate dependent claim 6.

**c) a “douche solution” (claims 18, 33, and 54)**

As to claims 18 and 33, Clayman additionally does not anticipate because it does not expressly or inherently disclose “a douche solution” (claims 18 and 33). The Examiner argues that a douche is simply a jet of liquid. Appellant notes that there is no disclosure in Clayman pertaining to any *jet of liquid* applied to any part of the body, vagina or otherwise.

**d) “a liquid carrier formulated for vaginal delivery” (claim 33)**

There is no indication in Clayman that any of the liquid formulations set forth therein are suitable for vaginal delivery. Clayman makes no mention of vaginal delivery. Rather, it is directed to treatment of disease of the oral cavity.

Further, the Examiner has cited no evidence to establish that any liquid carrier set forth in Clayman is a carrier formulated for vaginal delivery. It appears to Appellant that the Examiner argues that *any* formulation suitable for administration to the oral cavity is suitable for administration to the vagina. However, he has cited no evidence to support his assertion. The Examiner appears to be relying on facts within his own personal knowledge. In accordance with 37 C.F.R. §1.104(d)(2), Appellant calls for the affidavit of the Examiner to set forth those facts within his own personal knowledge which serve as the basis for this rejection.

The Examiner asserts that the burden is on Appellant to show that the prior art liquids set forth in Clayman are not suitable for administration as a douche to the vagina. However, Appellant is not required to meet this burden in the absence of any evidence from the Examiner to support his assertion that Clayman inherently discloses a douche solution suitable for vaginal administration.

### **3. Conclusion**

In view of the above, it is respectfully submitted that Clayman fails to expressly or inherently anticipate the claimed invention. Therefore, it is respectfully requested that the Board reverse the rejection of claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 under 35 U.S.C. §102(b) based on Clayman.

#### **B. Rejection of Claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54 Under 35 U.S.C. §102(b) as Being Anticipated by RAC, as Evidenced by Oda *et al.* and Flaitz *et al.***

##### **1. RAC Does Not Expressly or Inherently Disclose Papillomavirus-Transformed Cells**

As is the case with Clayman, RAC does not anticipate the claims because it fails to expressly or inherently describe each limitation of the claimed invention. See *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d at 631. The Examiner admits that RAC does not disclose papilloma virus infection in cells in the lesion, and thus concedes that there is no express anticipation. He argues that this characteristic is inherent in view of Oda *et al.* and Flaitz *et al.*, and that one of ordinary skill in the art of oral cancer would have been aware that treatment of hyperplastic lesions as described in RAC would necessarily involve treatment of hyperplastic lesions that comprise HPV infected cells.

For the reasons set forth above, the Examiner's interpretation of inherent anticipation is incorrect. Inherent anticipation arises when "the prior art necessarily functions in accordance



with, or includes, the claimed limitations.” *Atlas Powder Co.*, 190 F.3d at 1347. The Examiner has established, by citing Oda and Flaitz, that *not every malignancy or premalignancy of the cervix or oral cavity involves cells that are infected with HPV*. In fact, a substantial fraction of such lesions are not infected with HPV. Thus, he has established that it is *not necessary* for a malignancy or premalignancy of the cervix or oral cavity to include cells infected with HPV.

Furthermore, as set forth above, “inherency ... *may not be established by probabilities or possibilities.*” *MEHL/Biophile Int’l Corp. v. Milgraum*, 192 F.3d at 1365. The Examiner argues that “*it may be probable* that a premalignancy or cancer in one given individual would include HPV infected cells, the evidence presented in Oda and Flaitz show that one can be certain that in the population of individuals with oral or cervical cancer who are to be treated with the method of Clayman, RAC minutes, or Nielsen, half or most will have HPV infected cells in the premalignancy or cancer.” Office Action dated April 14, 2006 (emphasis added). In essence, he argues that the existence of a possibility that such a lesion might include HPV infected cells is sufficient to establish inherent anticipation. However, as discussed above, *occasional results are not inherent*. Without an inherent teaching regarding HPV-transformed cells, there can be no anticipation.

**2. RAC Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, 33, and 54**

For the same reasons discussed above pertaining to Clayman, the discussion of which is herein incorporated into this section, RAC does not anticipate claims 4, 6, 18, 33, or 54 because it does not expressly or inherently disclose “a keratinocyte,” (claim 4), “a skin cell” (claim 6), a “douche solution” (claims 18, 33, and 54), or “a liquid carrier formulated for vaginal delivery” (claim 33).

### 3. Conclusion

Therefore, in view of the above, it is respectfully submitted that RAC fails to expressly or inherently anticipate claims 1-12, 15, 18, 23-28, 33, 38-48, 51, and 54. Therefore, it is respectfully requested that the Board reverse this rejection.

#### C. Rejection of Claims 1-14, 19-29, 38-50, and 55-60 Under 35 U.S.C. §102(b) as Being Anticipated by Nielsen as evidenced by Oda *et al.* and Flaitz *et al.*

##### 1. Nielsen Does Not Expressly or Inherently Disclose HPV-Transformed Cells

Nielsen is said to describe the treatment of cancer in general, including cervical cancer and head and neck cancer, by a combination of p53 gene therapy and gemcitabine chemotherapy. It is said to disclose topical delivery, such as to a surgical wound following tumor resection. The Examiner, who admits that Nielsen does not mention papilloma virus infection of cells of the lesions, again relies on Oda *et al.* and Flaitz *et al.* to support an argument that Nielsen inherently anticipates the claims at issue in this rejection.

As with Clayman and RAC, the Examiner's interpretation of inherent anticipation is incorrect. The discussion above regarding why Clayman and RAC do not inherently anticipate the claims is herein incorporated into this section. As discussed above, *occasional results are not inherent*. Without an inherent teaching regarding HPV-transformed cells, there can be no anticipation.

##### 2. Nielsen Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, 33, and 54

Nielsen does not anticipate claims 4 or 6 because it does not expressly or inherently disclose "a keratinocyte," (claim 4), or "a skin cell" (claim 6).

### **3. Conclusion**

Therefore, in view of the above, it is respectfully submitted that Nielsen fails to expressly or inherently anticipate claims 1-14, 19-29, 38-50, and 55-60. Therefore, it is respectfully requested that the Board reverse this rejection.

#### **D. Rejection of Claims 1-15, 18-29, 33, 38-51, and 54-60 Under 35 U.S.C. 102(b) as Being Anticipated by El-Diery**

##### **1. El-Diery Does Not Anticipate Claims 1-15, 18-29, 38-51, and 54-60 Because it Does Not Expressly or Inherently Disclose Any Method that Involves Administration of a Polynucleotide Encoding a p53 Polypeptide to an Papillomavirus-Transformed Cell**

El-Diery does not anticipate the claimed invention because it does not expressly or inherently disclose administration of a polynucleotide encoding a p53 polypeptide to a papillomavirus-transformed cell. The Examiner argues that the recitation of "p73" in El-Diery is sufficient to anticipate the claims because El-Diery is said to disclose treatment of HPV-transformed cells using p73.

According to Appellant's specification, the term "p53" is intended to refer to "the exemplified p53 molecules as well as p53 homologues from other species." The exemplified p53 set forth in Applicant's specification is human p53. In particular, the Examples section of Appellant's specification sets forth results of studies pertaining to transduction of cell lines, including HPV-immortalized cell lines, with Ad-53. See specification, page 56, line 20 – page 61, line 31. The Ad-p53 is "Ad5CMV-p53; RPR/INGN 201," which was obtained from Introgen Therapeutics, Inc. Specification, page 58, lines 5-7. This particular construct includes a polynucleotide encoding a human p53 polypeptide.

El-Diery contains no disclosure pertaining to administration of human p53 to any papillomavirus-transformed cell. Nor does El-Diery disclose any p53 molecule from another species (*i.e.*, from a species other than human, such as a mouse p53).

In contrast, El-Diery teaches that p73 is structurally and functionally dissimilar to p53. For example, El-Diery indicates that unlike p53, p73 is not targeted for degradation in Ad-E6 infected cancer cells. El-Diery, page 5, lines 31-32.

Additionally, Appellant has submitted the Declaration of Louis Zumstein, PhD, under 37 C.F.R. §1.132 as evidence that El-Diery does not anticipate the claimed invention (**Exhibit 8**). In accordance with the requirement of 37 C.F.R. §1.132, Appellant has provided in their declaration a showing of evidence of such character and weight as to establish that El-Diery fails to anticipate the claimed invention within the meaning of 35 U.S.C. §102(b).

Dr. Zumstein, a person of skill in the art with over 13 years of experience in the biotechnology field, has indicated that, in his opinion, El-Diery does not include anticipate the claimed invention. He bases this opinion on the fact that there are characteristics that distinguish p53 and p73.

More specifically, while p73 and p53 do share some similar functions, and share some sequence similarities, there are important characteristics that distinguish the two proteins. For example, in contrast to p53 deficient mice, those mice lacking p73 show no increased susceptibility to spontaneous tumorigenesis. Additionally, p73 is not activated by DNA damage, unlike p53.

Declaration of Luis Zumstein, PhD, ¶ 6.

Therefore, because El-Diery does not disclose treatment of papilloma-virus transformed cells with “p53,” it fails to anticipate the claimed invention.

The Examiner cites Kaghad *et al.* (*Cell*, 90:809-819, 1997; **Exhibit 9**) as supporting his assertion that El-Diery anticipates the claimed invention. Kagdad *et al.* describes the sequence of p73. Although some sequence similarity of p73 to p53 is described in Kagdad *et al.*, it makes clear that there are substantial sequence dissimilarities as well. Further, regardless of some degree of sequence similarity, it is pointed out that “it is not obvious that p73 and p53 share

common functions.” See page 814, right column, first paragraph. Thus, Kagdad *et al.* discloses that p73 and p53 are structurally and functionally distinct, which further supports that El-Deiry does not anticipate the claimed invention.

**2. El-Deiry Does Not Expressly or Inherently Disclose Limitations of Dependent Claims 4, 6, 18, and 54**

Regarding dependent claims, El-Deiry additionally does not anticipate claims 4, 6, 18, 33, and 54 because it does not expressly or inherently disclose “a keratinocyte,” (claim 4), “a skin cell” (claim 6), a “douche solution” (claims 18, 33, and 54), or “a liquid carrier formulated for vaginal delivery” (claim 33). The Examiner has made no comment on the record regarding these deficiencies of El-Deiry

**3. El-Deiry Does Not Expressly or Inherently Anticipate Claim 33**

Regarding independent claim 33, pertaining to a douche solution for inhibiting the growth of a papillomavirus-transformed cell in a hyperplastic lesion in a subject, El-Deiry does not anticipate because it does not expressly or inherently disclose a “douche solution” or “a liquid carrier formulated for vaginal delivery.”

**4. Conclusion**

In view of the above, it is respectfully submitted that El-Deiry does not anticipate any of claims 1-15, 18-29, 33, 38-51, and 54-60 under 35 U.S.C. §102(b). Therefore, it is respectfully submitted that this rejection should be withdrawn.

**E. Rejection of Claims 16, 17, 31, 32, 52, and 53 as Being Unpatentable Under 35 U.S.C. §103(a) Over Either RAC as Evidenced by Oda *et al.* and Flaitz *et al.*, or El-Deiry in View of Zhang *et al.***

In order to establish a *prima facie* case of obviousness, three basic criteria must be met:

(1) the prior art reference (or references when combined) must teach or suggest all the claim

limitations; (2) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (3) there must be a reasonable expectation of success. *Manual of Patent Examining Procedure* § 2142. See also *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991) (emphasizing that the teaching or suggestion to make the claimed combination and the reasonable expectation of success must be both found in the prior art, and not based on Appellant's disclosure). It is important to note that all three elements must be shown to establish a *prima facie* case of obviousness. Thus, if one element is missing, a *prima facie* case of obviousness does not exist.

The Examiner has not met the PTO's burden of establishing a *prima facie* case of obviousness because he has not shown that the cited combination of references teach or suggest each limitation of the claimed invention. In particular, none of the cited references teach or suggest topically applying to a papillomavirus-transformed cell in a hyperplastic lesion in a subject a growth-inhibiting amount of a polynucleotide encoding a p53 polypeptide. As set forth above, RAC fails to teach or suggest any lesion that includes papillomavirus-transformed cells. Further, El-Deiry fails to teach or suggest a polynucleotide encoding human p53 or a p53 from another species. Further, El-Deiry provides no disclosure pertaining to topical application of any polynucleotide to papillomavirus-transformed cells. Zhang *et al.* fails to remedy the deficiency of RAC and El-Deiry because it is only cited as teaching a flavorant.

Further, the Examiner has failed to establish a *prima facie* case of obviousness because he has not set forth any suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine reference teaches. *Manual of Patent Examining Procedure* § 2142. See also *In re*

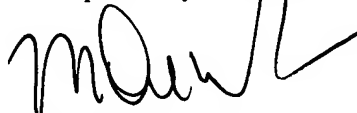
*Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991). No such teaching or suggestion has been set forth by the Examiner. It is the Examiner's responsibility to show that some objective teaching or suggestion in the applied prior art, or knowledge generally available [in the art] would have led one of ordinary skill in the art to combine the references to arrive at the claimed invention. *Pro-Mold & Tool Co. v. Great Lakes Plastics, Intl*, 745 F.3d 1568, 1573, 37 USPQ2d 1626, 1629 (Fed. Cir. 1996). In the absence of such a teaching or suggestion, there can be no *prima facie* case of obviousness.

In view of the above, the Examiner has failed to meet his burden of establishing a *prima facie* case of obviousness. Therefore, it is respectfully requested that this rejection should be withdrawn.

#### VIII. CONCLUSION

It is respectfully submitted, in light of the above, that none of the pending claims are properly rejected. Reversal of the pending grounds for rejection is thus respectfully requested.

Respectfully submitted,



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Date: July 11, 2006



## APPENDIX 1: LISTING OF APPEALED CLAIMS

1. (Original) A method of inhibiting the growth of a papillomavirus-transformed cell in a hyperplastic lesion in a subject comprising topically administering to said lesion a growth inhibiting amount of a composition comprising (a) an expression cassette comprising a promoter, active in cells of said lesion, operably linked to a polynucleotide encoding a *p53* polypeptide, and (b) a pharmaceutical preparation suitable for topical delivery, wherein expression of said *p53* polypeptide inhibits growth of said cell.
2. (Original) The method of claim 1, wherein said subject is a mammal.
3. (Original) The method of claim 2, wherein said mammal is a human.
4. (Original) The method of claim 1, wherein said cell is a keratinocyte.
5. (Original) The method of claim 1, wherein said cell is an epithelial cell.
6. (Original) The method of claim 1, wherein said cell is a skin cell.
7. (Original) The method of claim 1, wherein said cell is a mucosal cell.
8. (Original) The method of claim 1, wherein said papillomavirus is a human papillomavirus.
9. (Original) The method of claim 1, wherein said lesion is selected from the group consisting of a squamous cell hyperplastic lesion, premalignant epithelial lesion, psoriatic lesion, cutaneous wart, periungual wart, anogenital wart, epidermodysplasia verruciformis, an intraepithelial neoplastic lesion, focal epithelial hyperplasia, conjunctival papilloma, conjunctival carcinoma, or squamous carcinoma lesion.



10. (Original) The method of claim 1, wherein said expression cassette is carried in a viral vector.
11. (Original) The method of claim 10, wherein said viral vector is an adenoviral vector, a retroviral vector, an adeno-associated viral vector, a vaccinia viral vector or a pox viral vector.
12. (Original) The method of claim 10, wherein said viral vector is an adenoviral vector.
13. (Original) The method of claim 1, wherein said expression cassette is carried in a nonviral vector.
14. (Original) The method of claim 13, wherein said non-viral vector is a lipid.
15. (Original) The method of claim 1, wherein said composition is formulated as a mouthwash or mouthrinse.
16. (Original) The method of claim 15, wherein said mouthwash further comprises a flavorant.
17. (Original) The method of claim 16, wherein said flavorant is selected from the group comprising one or more flavor components selected from wintergreen oil, oregano oil, bay leaf oil, peppermint oil, spearmint oil, clove oil, sage oil, sassafras oil, lemon oil, orange oil, anise oil, benzaldehyde, bitter almond oil, camphor, cedar leaf oil, marjoram oil, citronella oil, lavender oil, mustard oil, pine oil, pine needle oil, rosemary oil, thyme oil, cinnamon leaf oil, and mixtures thereof.
18. (Original) The method of claim 1, wherein said composition is formulated as a douche solution.
19. (Original) The method of claim 1, wherein said composition is formulated as an ointment or salve.

20. (Original) The method of claim 1, wherein said composition is formulated as a cream for topical, anal or vaginal delivery.

21. (Original) The method of claim 1, wherein said composition is formulated as a spray or aerosol.

22. (Original) The method of claim 1, wherein said composition is formulated as a suppository for anal or vaginal delivery.

23. (Original) The method of claim 1, wherein the promoter is a constitutive promoter, an inducible promoter or a tissue specific promoter.

24. (Original) The method of claim 1, wherein inhibiting growth comprises in slowing or halting the growth of said lesion.

25. (Original) The method of claim 1, wherein inhibiting growth comprises a reduction in the size of said lesion.

26. (Original) The method of claim 1, wherein inhibiting growth comprises induction of apoptosis said cells of said lesion.

27. (Original) The method of claim 1, wherein inhibiting growth comprises induction of an immune response against said cells of said lesion.

28. (Original) The method of claim 1, further comprising subjecting said subject to a secondary anti-hyperplastic therapy.

29. (Original) The method of claim 28, wherein said secondary anti-hyperplastic therapy is chemotherapy, radiotherapy, immunotherapy, phototherapy, cryotherapy, toxin therapy, hormonal therapy or surgery.

30. (Canceled)

31. (Previously Presented) A mouthwash for inhibiting the growth of a papillomavirus-transformed cell in a hyperplastic lesion in a subject comprising (a) an expression cassette comprising a promoter operably linked to a polynucleotide encoding a *p53* polypeptide, (b) a liquid carrier formulated for oral delivery, and (c) a flavorant.

32. (Original) The mouthwash of claim 31, wherein said flavorant is selected from the group comprising one or more flavor components selected from wintergreen oil, oregano oil, bay leaf oil, peppermint oil, spearmint oil, clove oil, sage oil, sassafras oil, lemon oil, orange oil, anise oil, benzaldehyde, bitter almond oil, camphor, cedar leaf oil, marjoram oil, citronella oil, lavender oil, mustard oil, pine oil, pine needle oil, rosemary oil, thyme oil, cinnamon leaf oil, and mixtures thereof.

33. (Original) A douche solution for inhibiting the growth of a papillomavirus-transformed cell in a hyperplastic lesion in a subject comprising (a) an expression cassette comprising a promoter operably linked to a polynucleotide encoding a *p53* polypeptide, and (b) a liquid carrier formulated for vaginal delivery.

34-37. (Canceled)

38. (Original) A method of suppressing or preventing papillomavirus-mediated transformation of a cell in a subject comprising administering to said cell a composition comprising (a) an expression cassette comprising a promoter, active in said cell operably linked to a polynucleotide encoding a *p53* polypeptide, and (b) a pharmaceutical preparation suitable for topical delivery, wherein expression of said *p53* suppresses said transformation.

39. (Original) The method of claim 38, wherein said cell is a keratinocyte.

40. (Original) The method of claim 38, wherein said subject is a human.

41. (Original) The method of claim 38, wherein said subject is a human at risk of developing an oral hyperplastic lesion.

42. (Original) The method of claim 41, wherein said human at risk of developing an oral hyperplastic lesion is a human with a history of a previous oral hyperplastic lesion.

43. (Original) The method of claim 42, wherein said previous oral hyperplastic lesion is comprised of cells selected from the group consisting of premalignant epithelial cells, squamous intraepithelial neoplastic cells, squamous hyperplastic cells, and squamous cell carcinoma cells.

44. (Original) The method of claim 41, wherein said oral hyperplastic lesion is comprised of cells transformed by a papillomavirus.

45. (Original) The method of claim 44, wherein said papillomavirus is a human papillomavirus.

46. (Original) The method of claim 38, wherein said expression cassette is carried in a viral vector.

47. (Original) The method of claim 46, wherein said viral vector is an adenoviral vector, a retroviral vector, an adeno-associated viral vector, a vaccinia viral vector or a pox viral vector.

48. (Original) The method of claim 46, wherein said viral vector is an adenoviral vector.

49. (Original) The method of claim 38, wherein said expression cassette is carried in a nonviral vector.

50. (Original) The method of claim 49, wherein said nonviral vector is a lipid.

51. (Original) The method of claim 38, wherein said composition is formulated as a mouthwash or mouth rinse.

52. (Previously Presented) The method of claim 51, further comprising a flavorant.

53. (Previously Presented) The method of claim 52, wherein said flavorant is selected from the group comprising one or more flavor components selected from wintergreen oil, oregano oil, bay leaf oil, peppermint oil, spearmint oil, clove oil, sage oil, sassafras oil, lemon oil, orange oil, anise oil, benzaldehyde, bitter almond oil, camphor, cedar leaf oil, marjoram oil, citronella oil, lavender oil, mustard oil, pine oil, pine needle oil, rosemary oil, thyme oil, cinnamon leaf oil, and mixtures thereof.

54. (Original) The method of claim 38, wherein said composition is formulated as a douche solution for vaginal delivery.

55. (Original) The method of claim 38, wherein said composition is formulated as a suppository for anal or vaginal delivery.

56. (Original) The method of claim 38, wherein said composition is formulated as an ointment or salve.

57. (Original) The method of claim 38, wherein said composition is formulated as a cream for topical, anal or vaginal delivery.

58. (Original) The method of claim 38, wherein said composition is formulated as a spray or aerosol.

59. (Original) The method of claim 38, wherein said composition is formulated as a pill or capsule.

60. (Original) The method of claim 38, wherein said composition is formulated for timed-release.

## APPENDIX 2: EVIDENCE APPENDIX

1. Clayman, G. ("Clinical protocol for wild type p53 gene induction in premalignancies of squamous epithelium of the oral cavity via an adenoviral vector," Investigator Gary Clayman, Sponsor Introgen Therapeutics, PowerPoint presentation, March 2001): cited by Appellant in Information Disclosure Statement filed August 16, 2004.
2. Oda *et al.* (*Carcinogenesis* 17(9):2003-2008, 1996): cited by Appellant in Information Disclosure Statement filed August 16, 2004.
3. Flaitz *et al.* (*Oral Oncol.* 34:448-453, 1998): cited by Examiner in Office Action Dated November 17, 2005.
4. Recombinant DNA Advisory Committee (RAC) (Minutes of Meeting dated March 8, 2001, U.S. Dept. of Health and Human Services): cited by Examiner in Office Action Dated November 17, 2005.
5. Nielsen *et al.* (U.S. Patent App. Pub. No. 2001/0044420): cited in by Examiner in Office Action Dated November 17, 2005.
6. El-Deiry (WO 99/66946): cited by Examiner in Office Action Dated November 17, 2005.
7. Zhang *et al.* (WO 00/29024): cited by Examiner in Office Action Dated November 17, 2005.
8. Declaration of Louis Zumstein, PhD (with Appendix A): filed by Appellant on February 17, 2006 in Response to Office Action Dated November 17, 2005.
9. Kaghad *et al.* (*Cell*, 90:809-819, 1997): cited by Examiner in Office Action Dated April 14, 2006.

### **APPENDIX 3: RELATED PROCEEDINGS APPENDIX**

None.



# **EXHIBIT 1**

# **Clinical Protocol for Wild Type p53 Gene Induction in Premalignancies of Squamous Epithelium of the Oral Cavity via an Adenoviral Vector**

**Primary Investigator: Gary Clayman M.D.**

**Sponsor: Introgen Therapeutics, Inc.**



**INTROGEN**

## **RPR/INGN 201 (Ad5CMV-p53)**

- Adenoviral vector expressing p53
- Completely sequenced
- CGMP Manufacturing in validated clean-room facility by experienced personnel
- Final product testing in compliance with CGMP and current industry practice



**INTROGEN**

# **Previous Human Exposure to RPR/INGN 201 (Ad5CMV-p53)**

- In use in human clinical trials since 1995
- Over 480 people exposed by various routes of administration
- Head & Neck phase III trials actively ongoing
- Phase I & II trials ongoing in additional indications
- Large safety database for this material



**INTROGEN**

## Protocol Description

- Combination of intramucosal injection of RPR/INGN 201 (Ad5CMV-p53) in area of lesion followed by a series of oral swishes with same
- Patients participate in protocol for 6 months, each 1 month cycle begins with 5 days of exposure to RPR/INGN 201 (Ad5CMV-p53)



**NITROGEN**

## **Protocol Description: Day 1**

- Lesion will be injected with RPR/INGN 201 (Ad5CMV-p53)
- Biopsy of lesion and contralateral region after 2 hours
- 10% acetic acid rinse for 2 minutes
- RPR/INGN 201 (Ad5CMV-p53) swish for 30 minutes



**INTROGEN**

## **Protocol Description: Day 2-4**

- 10% acetic acid rinse
- RPR/INGN 201 (Ad5CMV-p53) swish and spit
- Minimum 2 hour observation
- Repeat rinse and swish/spit



**INTROGEN**

## **Protocol Description: Day 5**

- 10% acetic acid rinse
- RPR/INGN 201 (Ad5CMV-p53) swish and spit
- Minimum 2 hour observation
- Biopsy of lesion and contralateral region
- Repeat rinse and swish/spit



**INTROGEN**



# **Special Protocol Testing Summary**

## **Pre Treatment**

- p53 genotype of microdissected lesion
- HPV of microdissected lesion
- H&E on all biopsy specimens
- TUNEL on lesion & contralateral region
- CAR on lesion & contralateral region
- p53 IHC on contralateral region only
- Antibody to serotype 5 adenovirus in serum



**INTROGEN**

# **Special Protocol Testing Summary**

## **Post Treatment**

- H&E on lesion & contralateral region
- TUNEL lesion & contralateral region
- CAR lesion & contralateral region
- p53 IHC on contralateral region



**INTROGEN**

# **RAC Reviewer Protocol Questions**

- Risk to benefit ratio
- Efficacy evaluations
- Safety of acid rinse
- 30 minute duration of swish
- Consenting process



**INTROGEN**

# Risk to benefit ratio



**INTROGEN**

# Disease Characteristics

- Patients diagnosed with a preneoplastic lesion of the oral cavity will progress to a malignant state, most within six months.
- Surgery is frequently not effective because of diffuse, multifocal nature of these lesions.
- Patients eligible for this trial will have failed other approaches being tested.



**INTROGEN**

# **Why this study, Why this patient population**

- Two Case Studies



**INTROGEN**

## **Case Study #1**

- Prior history of multiple surgical procedures for removal of retromolar area lesions
- 1986 diagnosis mild dysplasia in multiple locations in oral cavity
- 1988 treatment with topical Retin A gel, discontinued because of patient discomfort



**INTROGEN**

## **Case Study #1 (continued)**

- Aug 1993 carcinoma in situ, surgically removed, margins pathologically free of cancer
- Nov 1993 moderate to severe dysplasia in same area, lesion removed by laser ablation



**INTROGEN**



## **Case Study #1 (continued)**

- Feb 1994 moderate dysplasia in same area
- July 1994 continued inflammation & dysplasia, Biochemoprevention therapy
- Dec 1994 continued inflammation & dysplasia



**INTROGEN**

## **Case Study #1 (continued)**

- April 1996 Invasive squamous carcinoma
- May 1996 Resection of major portions of oral cavity, margins pathologically free of cancer
- May 1996 Post operative radiation therapy
- July 1996 Extensive local, facial and infratemporal fossa recurrence treated with chemotherapy and radiotherapy
- Sep 1996 Patient died of disease



**INTROGEN**

## **Case Study #2**

- June 1992 Premalignant left oral lesion surgically removed, recurrence within six months
- Feb 1994 Laser ablation to remove left oral tongue premalignant lesion, recurrence within six months
- May 1996 biopsy shows mild to moderate dysplasia + hyperkeratosis of left oral tongue
- July 1996 Biochemoprevention studies initiated



**INTROGEN**

## Case Study #2 (continued)

- Jan 1997 continued left oral tongue dysplasia
- Dec 1997 new lesion on right oral tongue; surgery to remove part of tongue, floor of mouth and neck. Laser ablation to clear a larger area. Margins free. Patient ineligible for biochemoprevention since he developed lesion while on that attempted prevention approach
- Feb 2001 Patient has dysplasia in two areas of oral cavity



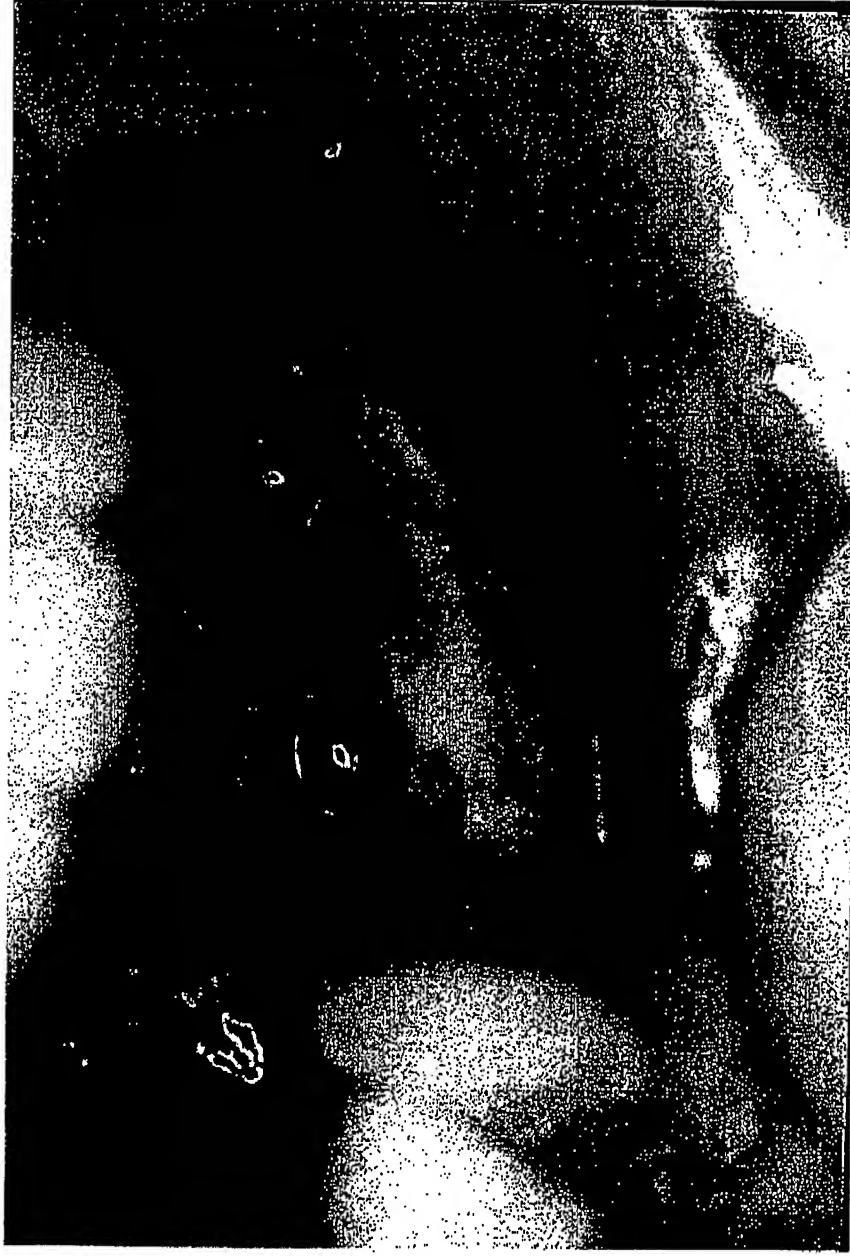
**INTROGEN**

# Diagnosis: Leukoplakia without dysplasia of the Oral Cavity



**INTROGEN**

# Diagnosis: Erythroplasia with severe dysplasia of the Oral Cavity



**INTROGEN**

# **Diagnosis: Preneoplastic Lesion of the Oral Cavity**



**INTROGEN**

# Progression to Malignant Lesion



**NITROGEN**

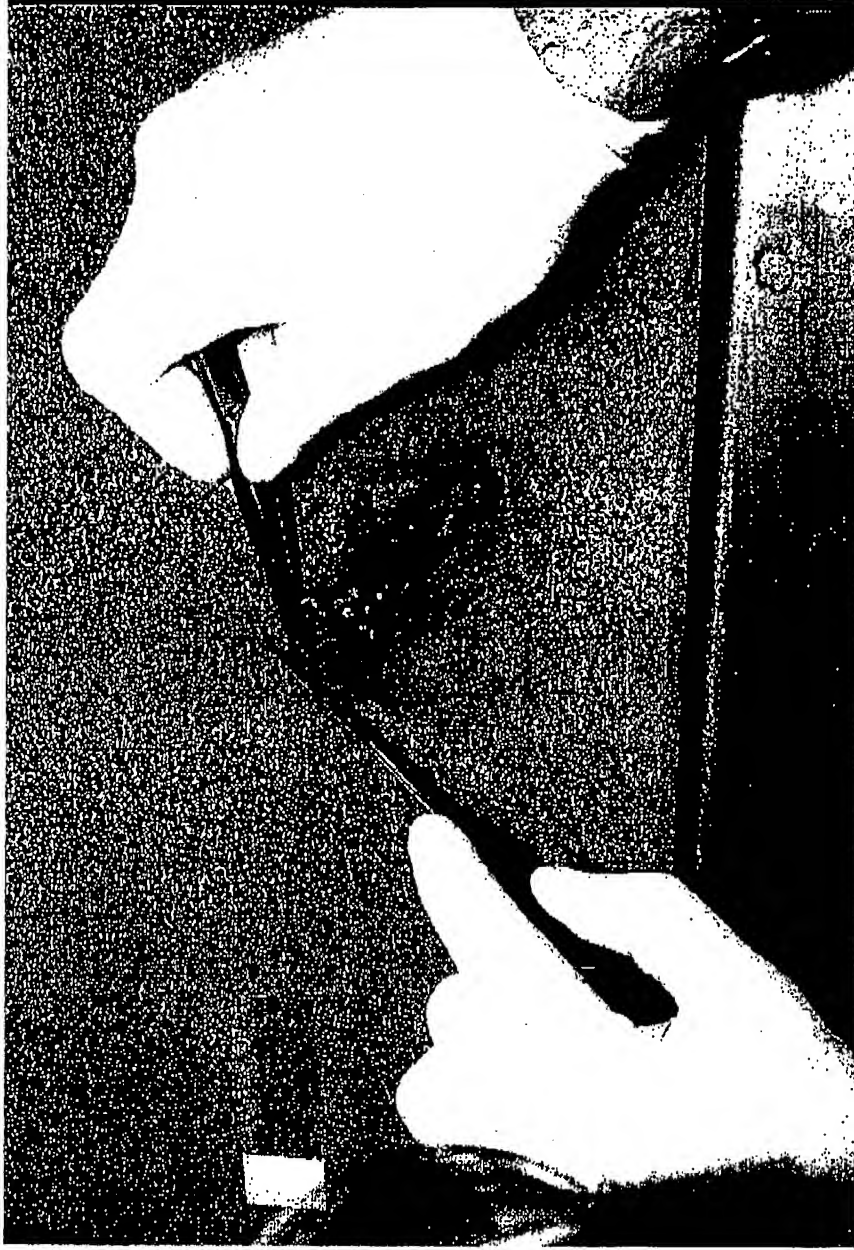


# **Surgical removal of carcinoma *in situ***



**INTROGEN**

# Comprehensive pathologic analysis of premalignant lesion



**INTROGEN**

# **Extensive surgical margin required for premalignant lesion**



**NITROGEN**

# **Efficacy Evaluation versus Protocol Goals for a Phase I/II Clinical Trial**

- As with all Phase I/II clinical trials, the primary objective is to evaluate safety
- Any scientific information gathered is a secondary objective
- Any efficacy information that comes from a phase I/II is a secondary objective



**INTROGEN**

# **Previous Routes of Administration for RPR/INGN 201 (Ad5CMV-p53)**

- Intratumoral in Head & Neck
- Lavage in Lung
- Intratumoral in Lung
- Intratumoral in prostate, ovary, breast,  
bladder
- IV



**INTROGEN**

## **Safety of Acid Rinse**

- 10% acetic acid approximately the strength of household vinegar (6%)



**INTROGEN**

# pH Comparison

Item	FDA or USP acceptable pH range	Example of tested pH
10% acetic acid	N/A	2.2
Coca Cola	N/A	2.3
Sauerkraut	3.4-3.6	3.3
Grapefruit juice	3.0-3.3	3.5



**INTROGEN**

# **Questions on 30 minute duration of swish**

- Route of administration
- Safety for other tissues
- Potential for aspiration



**NITROGEN**



## **30 minute duration of swish**

- Common route of administration
- Used for Tingel, approved fluoride compound
- Not uncomfortable for patients
- Will be performed under clinical observation



**INTROGEN**

# **Previous Routes of Administration to Head & Neck**

- Intratumoral and intramucosal injections in multiple head and neck sites
- Intraoperative injection and lavage into surgical beds
- Intraoperative injection into mucosal margins following cancer removal



**INTROGEN**

# **Previous of Administration to Lung**

- Bronchoalveolar lavage in Lung
- Intratumoral in Lung



**INTROGEN**

# **Additional Previous Routes of Administration**

- Intraprostatic injection
- Intervescicle in bladder
- Intravenous
- Intraperitoneal in ovary
- Intratumoral in breast



**INTROGEN**

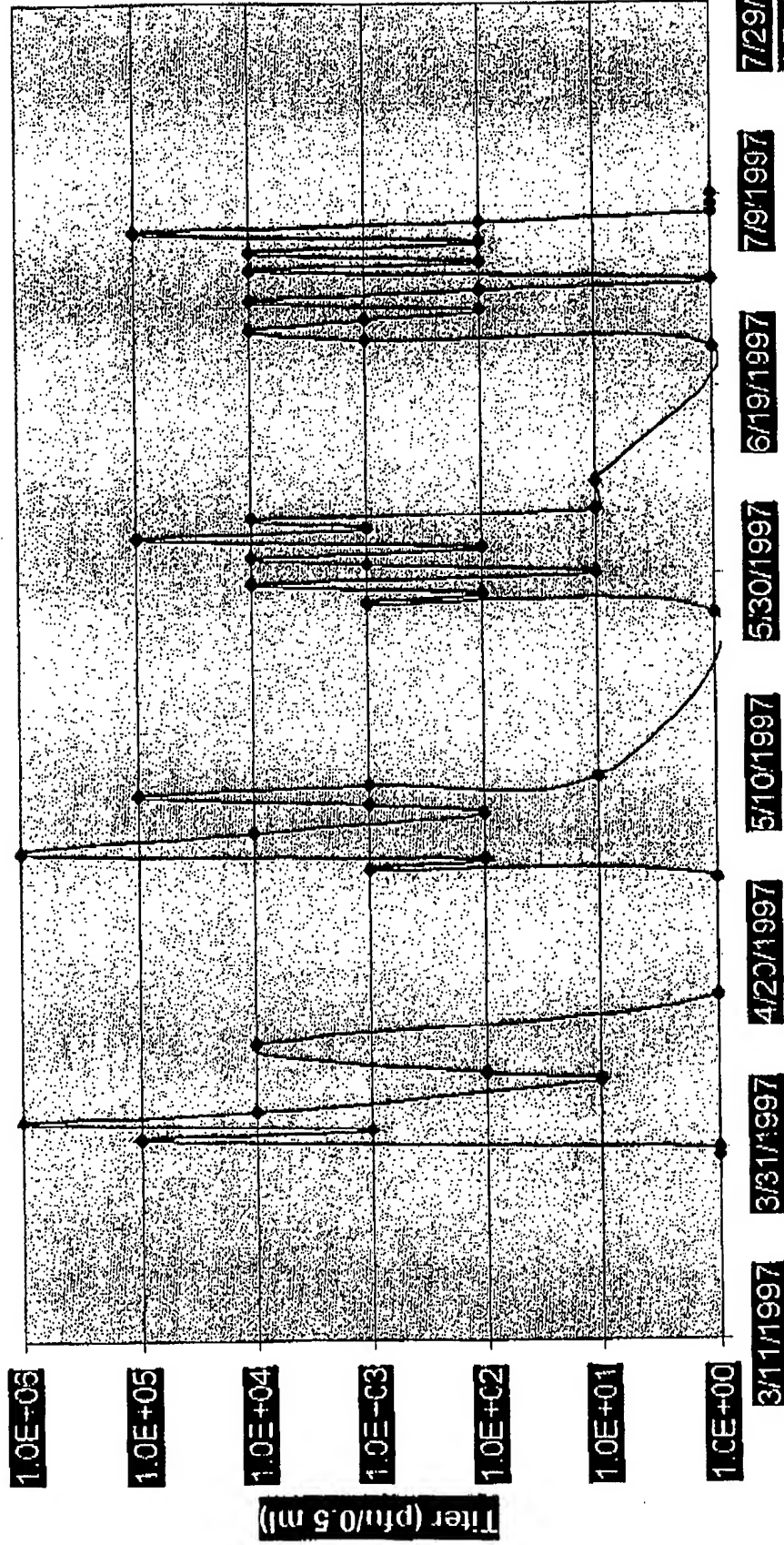
# **RPR/INGN 201 (Ad5CMV-p53): Safe for normal tissue?**

- Not toxic to non-malignant structures
- Post-surgical injection into healthy surgical beds of the Head and Neck and oral cavity
- Intraprostatic injection exposed normal prostate tissue
- Lavage of cerebral cortex exposed normal brain tissue
- All routes tested thus far are well tolerated



**INTROGEN**

# RPR/INGN 201 in Upper Aerodigestive Tract Secretions Following Intratumoral Administration



# Post surgical injection into healthy surgical bed



**INTROGEN**

## **Informed Consent**

- Viewed by investigator and sponsor of this trial as a process beyond obtaining appropriate signature
- Witness required by IRB to observe the informed consent process
- Important gene transfer considerations included



**INTROGEN**



# **EXHIBIT 2**

# Chromosomal abnormalities in HPV-16-immortalized oral epithelial cells

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<sup>5</sup>To whom correspondence should be addressed

Human papilloma virus (HPV) type 16 has an established association with anogenital carcinoma, and to some extent with human oral squamous cell carcinoma. We hypothesize that HPV type 16 is capable of inducing chromosomal and cell cycle changes in cultured oral epithelial cells. Normal human oral epithelial cells were immortalized with recombinant retrovirus containing the E6/E7 open reading frames of HPV type 16. These cells have been in culture for more than 350 passages and over 4 years. Flow cytometry demonstrated an average of 42% nuclear aneuploidy in HPV 16-immortalized cells; 16% in normal controls (probably tetrasomy). Cytogenetic analysis demonstrated significant progression of chromosomal abnormalities. Cells at early passage (p10) showed trisomy 20, with no other major changes. At passage 18, trisomy 1q and monosomy 13 were seen in addition to trisomy 20. At passage 61 there were two distinct cell populations ('a' and 'b'), with multiple chromosomal changes including trisomy 5q,14,20 in one line and 7p,9q,11q in the other. Both populations had monosomy 3p, with monosomy 8p in one population and monosomy 13 in the other. At passage 136, the cells were essentially identical to population 'b' of passage 61. At this passage, mutation of the p53 gene was detected at codon 273 of exon 8, with G to T conversion (Arg to Leu). This was absent in the normal cells from which this line was developed. Passage 262 contained the two major cell populations, each with a sub-group with additional chromosomal changes such as 10p monosomy. Cells from passages 217 and 305 were injected into nude mice a year apart. Both failed to produce tumors, as did normal cells. In conclusion, we present an HPV type 16-immortalized oral epithelial cell line (IHGK) with extensive and progressive chromosomal abnormalities, invasive growth in culture and yet no tumor formation in nude mice. We suggest that the question as to whether HPV alone can induce transformation is still open.

## Introduction

It is generally accepted that carcinogenesis is a multi-hit process, involving a number of aberrant genetic changes and culminating in malignant transformation (1). There can be abnormal products or over-production of growth factors, recep-

tors or genes controlling signaling from cell surface to nucleus (2). Oncogenes are probably involved in both initiation and progression of neoplasia (3).

The role of human papilloma virus (HPV\*) in the pathogenesis of anogenital and oral cancer has been studied extensively at the clinical, epidemiological and experimental levels. HPV is a site-specific DNA virus that is known to infect the basal cell layer and replicate during epithelial cell differentiation. Of the more than 70 types of HPV that have been identified, 22 are related to malignant lesions that include cervical and oral cancers. Between 85 and 90% of cervical cancers and high-grade cervical intraepithelial neoplasia contain HPV DNA, with types 16 and 18 most common (4–7). Similarly, up to 90% of oral cancers have been reported to contain HPV DNA, again with types 16 and 18 predominating (8). E6 and E7 oncoproteins are consistently expressed in HPV-positive cervical carcinomas and cell lines (9–11). Their gene products are transforming proteins that can form complexes with both the retinoblastoma and p53 tumor-suppressor gene products (12,13).

HPV 'high risk' types are 16 and 18, while low risk types are 6b and 11. Types 16, 18 and 33 are most prevalent in malignant cells (14). Type 18 is generally associated with glandular tumors and type 16 with squamous tumors (15). Type 18 is associated with more aggressive cervical tumors than type 16 (15,16) and with younger age at diagnosis and greater frequency of lymph node metastasis (17).

Immortalization of cells following transfection with HPV 16 DNA is a reproducible phenomenon that occurs with a high frequency independent of the genetic characteristics of the host cells (18,19). While HPV DNA may persist in episome form in benign lesions, most tumors and tumor cell lines show single or multiple integrated copies of HPV 16 or 18 DNA (20–25). Only cell lines having HPV sequences integrated into cellular DNA become permanent lines, showing that genetic alterations caused by viral DNA integration are necessary for continuous growth (19). Immortalization of human cells by viral DNA is usually associated with aneuploidy and rearrangement of chromosomes (19,26–32).

The limited knowledge of the molecular and genetic events in human oral cancer, and its relationship to HPV, is partly due to the lack of a well-established suitable *in vitro* model (33). If the genetic and/or molecular markers of oral cancer are known, they can be detected by non-invasive sampling of epithelium such as oral scrapings. We present an *in vitro* model in which normal human oral epithelial cells have been immortalized with HPV type 16 DNA E6/E7 genes (33). Cell kinetics and chromosomal changes in the cell lines are presented. For brevity, we will refer to this cell line as immortalized human gingival keratinocytes (IHGK).

## Materials and methods

Chemicals were obtained from Sigma Chemical Co. (St Louis, MO) except where noted. Vitrogen (bovine dermal collagen) was purchased from Celtrix

\*Abbreviations: HPV, human papilloma virus; IHGK, immortalized human gingival keratinocytes; KBM, keratinocyte basal medium; PBS, phosphate-buffered saline; K-SFM, keratinocyte SFM.

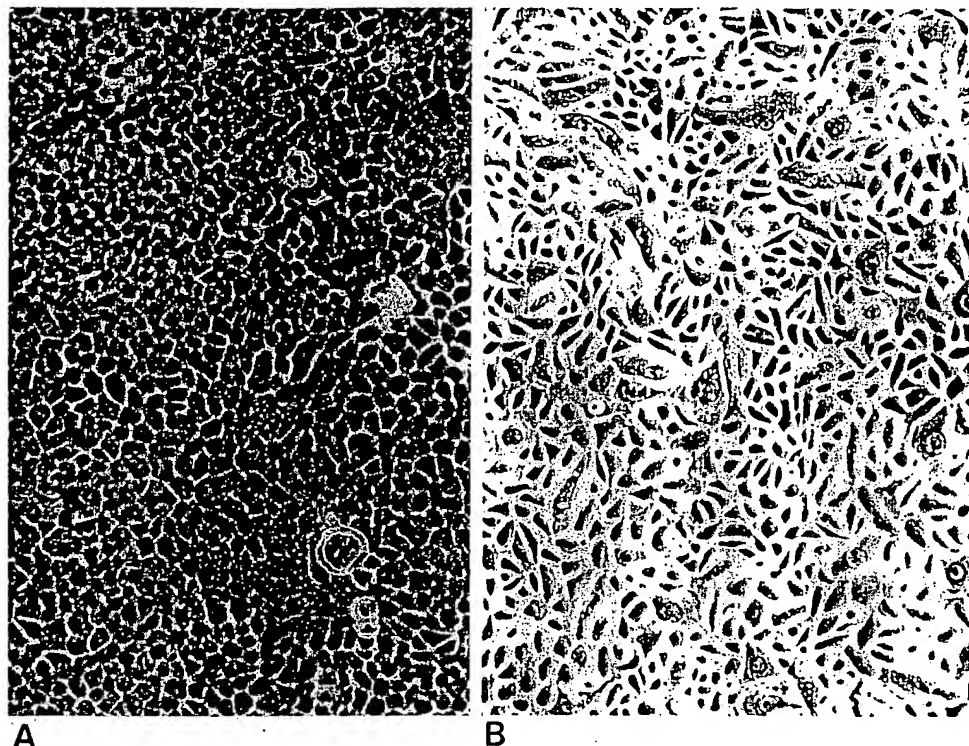


Fig. 1. Phase contrast microscopy of human oral epithelial cells in monolayer culture. (A) Normal cells. (B) HPV 16-immortalized cells.

Lab. (Palo Alto, CA). Falcon culture plates were from Becton Dickinson Co. (Franklin Lakes, NJ) and Transwell inserts from Costar Co. (Cambridge, MA).

#### Monolayer culture

We followed the procedure described by Oda and Watson (34). Specimens obtained from healthy patients undergoing surgery for impacted third molar removal were washed immediately with phosphate-buffered saline (PBS). After removing excess and damaged epithelium and connective tissue, the specimens were cut into small pieces and incubated overnight in Dispase II (Boehringer Mannheim, Mannheim, Germany) at a concentration of 4 mg/ml in PBS, with agitation, at 4°C. Surface epithelium was mechanically separated and trypsinized to dissociate the cells into a single cell suspension. The cells were centrifuged and resuspended in keratinocyte basal medium (KBM) with BEGM singlequats supplements (Clonetics, San Diego, CA). The parent population used for immortalization was derived from one healthy donor.

#### HPV 16 Immortalization

Normal epithelial cells at 50% confluence were transfected with HPV 16 E6/E7 open reading frames (ORFs) using a recombinant retroviral system, as previously described (35). Briefly, HPV E6/E7 ORFs were cloned into the murine-based retroviral vector LXSN. The constructs were transfected into a packaging cell line PA317 and recombinant retrovirus collected in the supernatant. The resulting vector, PLXSN, was used to infect the early passage oral epithelial cells. The cells were selected with G418 at 120 µg/ml and passaged in culture. Normal control and normal cells with PLXSN vector also were treated with G418. Over 95% of the HPV-treated cells were healthy after the G418 treatment. They were trypsinized and passaged twice while maintained on G418. The normal and PLXSN vector cells died around 72 to 96 h after treatment with G418 (35). Fifteen clones were selected at passage 3 after G418 selection. They were passaged and cryopreserved for further study. The parent population (IHGK) of immortalized cells were subsequently maintained in long-term culture with Keratinocyte SFM (K-SFM) medium with standard supplements (Gibco) and 0.05mM calcium chloride.

#### Flow cytometry

For staining and flow cytometry, the procedure of Rabinovitch (36) was followed. Cells at monolayer were trypsinized and cell pellets were then resuspended in 0.5 ml of ethidium bromide containing 0.1% Non idet P40 detergent. To this was added 0.1 ml of RNase. Samples were allowed to stand at room temperature for 20 min, after which 0.5 ml of mithramycin staining solution was added for 10 min. Then 0.11 ml of dimethylsulfoxide was added, the samples were evenly mixed and analyzed with an epifluorescence flow system designed by GÖHDE (ICP21; Phywe AG, Göttingen, West Germany; now Ortho Instruments, Westwood, MA).

#### Cytogenetic analysis

A single cell suspension was prepared from the monolayer cultures at the indicated passages, and cells were subcultured in K-SFM and placed onto clean microscope slides. Following 48 h subculture, colcemid (0.1 µg/ml) was added to the culture medium for 2 h prior to chromosome harvest. The latter was prepared in a standard fashion using KCl 0.075M as a hypotonic medium and methanol/acetic acid (3:1) as fixative. After staining by G-banding, 20–21 cells were completely analyzed for each cell line. Two to 20 cut karyotypes were established for each cell line. Karyotypes were described using the ISCN (International System for Human Genetics Nomenclature, 1985, 1991).

#### DNA Extraction

Digestion with proteinase K and phenol/chloroform extraction were used to prepare specimens for DNA analysis and for PCR amplification (37).

Detection of p53 mutation by SSCP-PCR and sequencing: PCR primers for p53 exons 5 to 8 were used in this study, as these exons were previously shown to have a high incidence of mutations (38).

PCR amplification was performed by the standard method with necessary modifications. Briefly, the PCR reaction mixture consisted of 25 µl reaction volume containing 0.25 µg genomic DNA, 10 mM Tris-HCl, pH 8.4, 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin, 100 mM dNTPs (10 mM dCTP), 75 ng of each primer, 5 µCi of alpha-[<sup>32</sup>P]dCTP (3000 Ci/mmol) and 1 U of Taq polymerase. After a brief spin, the reaction mixtures were heated in an automated thermal cycler (Perkin Elmer Corporation, Norwalk, Connecticut) for 35 cycles of amplification.

The PCR product was diluted 10-fold with SSCP running dye containing 20 mM EDTA (pH 8.0), 0.1% xylene cyanol, 95% deionized formamide and 0.1% bromophenol blue. The reaction was heated at 95°C for 5 min., immediately chilled on ice, and loaded onto a non-denaturing gel consisting of 8% acrylamide, 5% (v/v) glycerol and 0.5% TBE buffer. Samples were electrophoresed at room temperature in 0.5% TBE running buffer. After migration, the gels were dried on filter paper and exposed to Kodak XAR X-ray film at room temperature overnight without an intensifying screen. The C33A cell line DNA was used as a positive control, as it has been confirmed to have mutation of exon 8 (39). DNA from human placenta was used as a negative control for each analysis. Direct sequencing of single-stranded PCR products was carried out by Taq dideoxy Terminator Auto Cycle Sequencing System (Applied Biosystems). At least two independent PCR products were sequenced in each case.

#### Tumorigenicity

At passages 217 and 305, 10<sup>7</sup> HPV-immortalized human oral cells (in a total volume of 0.1 ml) were injected sub-cutaneously once into the backs of two

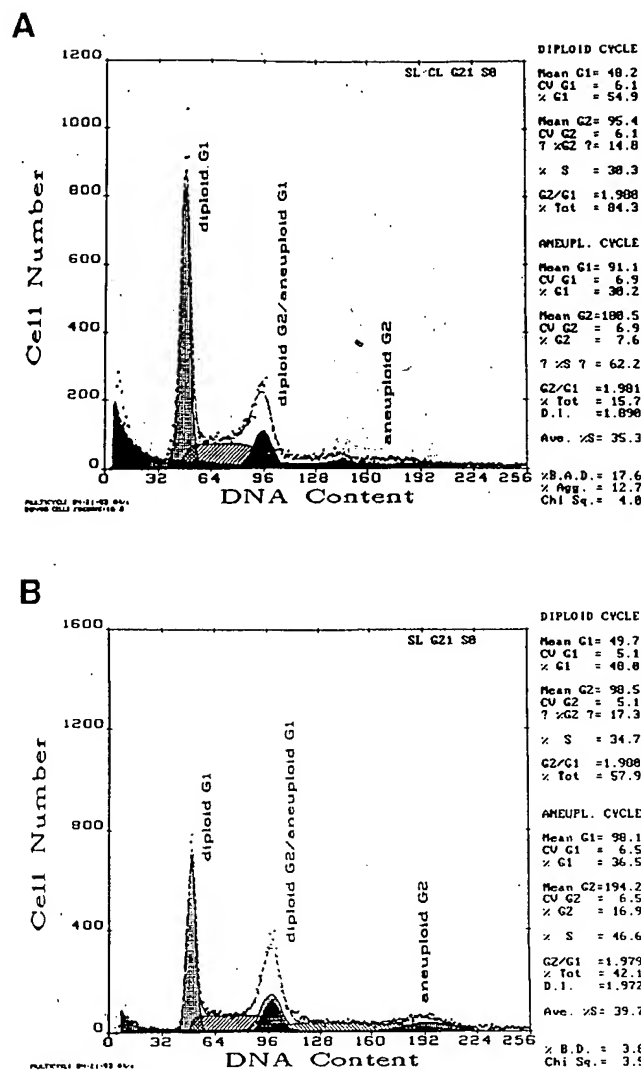


Fig. 2. Flow cytometry of human oral epithelial cells in monolayer culture. (A) Normal cells, (B) HPV 16-immortalized cells.

nude mice per group (strain nu/nu BALB/c, female, adult, 15–20 g). The mice were not subjected to X-irradiation prior to treatment, as the authors feel that this may lead to tumorigenicity of questionable origin (56). They were fed a standard rodent diet and observed on a weekly basis for tumors, in addition to being monitored for distress.

## Results

The IHGK cells have been in culture for over 4 years and more than 350 passages, while the normal control survive between seven and nine passages (33). By phase contrast microscopy (Figure 1) they are small, uniform and basaloid in morphology (A) while the normal control cells are heterogeneous with predominant basaloid cells interspersed with larger, more differentiated cells (B). By organotypic culture, the IHGK cells were pleomorphic with high mitotic activity and invading the underlying fibroblast/collagen matrix. The normal control cells maintained the usual pattern of cell maturation. When stained with keratin 19, an embryonal keratin found in premalignant and malignant epithelial cells (57), the IHGK cells stained strongly and uniformly positive while the normal control showed focal positive staining along the basal cell layer as expected in normal epithelial cells after birth (33).

By flow cytometry, the IHGK cells are 58% near-diploid and 42% near-tetraploid (Figure 2B), compared to the normal



Fig. 3. Example of G-banded karyotypes from passage 262. (A) is representative of population 'a'; (B) is representative of population 'b'.

control cells, which are 84% diploid and 16% tetraploid (Figure 2A). The karyotype of the normal cells was consistently normal, and the 16% aneuploidy is not unusual in cultured cells, and is probably due to tetraploidy.

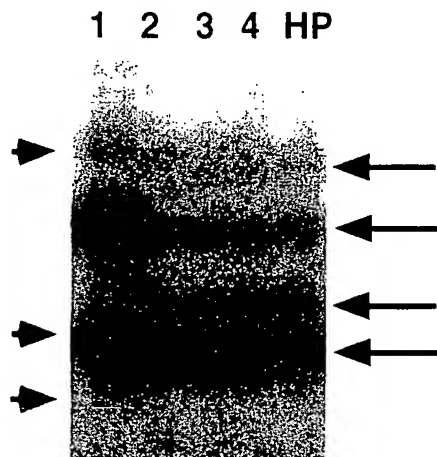
On cytogenetic analysis, the IHGK cells showed numerous chromosomal abnormalities, summarized in Tables I, and in Figures 3A and B. All 20 cells karyotyped at each passage showed similar abnormalities. Therefore, the karyotypes indicated are composite to reflect the common abnormalities. Most passages showed trisomy 20, however it was absent from population 'b' at passage 61, passage 136 and the 'b' population at passage 262. Monosomy 3p and 13 were noted in the middle passages (the latter indicating possible loss of Rb tumor suppressor gene) together with multiple chromosome rearrangements. The number of chromosomal abnormalities increased throughout, unlike the HPV-immortalized cell lines reported by Smith *et al.* (40). Cells at passage 61 clearly showed two chromosomally different cell populations, which persisted up to passage 262, although at passage 136 only one population (b) was observed. At passage 262, subgroups of populations 'a' and 'b' had developed with characteristic additional chromosomal changes such as monosomy 10p. The control normal cells, derived from the oral epithelium of a male and not infected with HPV, had a normal 46, XY karyotype.

## p53 mutations

With the SSCP-PCR technique, passage 136 cell line was screened for point mutations in exons 5–8 of the p53 gene.

**Table I.** Karyotypes and common chromosomal changes of HPV 16-immortalized oral epithelial cells at different passages with progression in culture

Cell Passage	Trisomy	Monosomy	Rearrangements
Passage 10: 45-49, XY, add (13)(p11), +20(cp20)	20		13p11
Passage 18: 44-48, XY, i(8q), +9, der(11)t(1;11)(q21;q23), -13, add (14)(p11), +16, add(19)(p13.3), +20, +22(cp20)	1q, 20	13	1q21,11q23
Passage 61:			
Population a: 45-47, XY, der(14)t(3;14)(p11;p11), +der(3)t(3;14)(q10;q10), der(8)t(8;21)(p11;q11), 14,+20,der(22)t(5;22)(q13;q13)(cp16)	5q, 14, 20	3p, 8p	3p11, 3q10, 5q13, 8p11, 14p11, 14q10, 21q11, 22q13
Population b: 43-44, XY, der(3)t(3;7)(q10;q10), der(16)t(9;16)(q11;q24),der(20)t(9;20)(q11;q13), add(9)(q12),-13, der(14)t(14;15)(q10;q10), -15, der(22)t(11;22)(q11;q13) (cp5)	7p, 9q, 11q	3p,13	3q10, 7q10, 9q11, 9q12, 11q11, 14q10, 15q10, 16q24, 20q13, 22q13
Passage 136: 45, XY, der(3)t(3;7)(q10;q10), add(9)(q12),-13, add(14)(p11), der(16)t(9;16)(q11;q24), der(20)t(9;20)(q11;q13),der (22)t(11;22)(q11;q13) (cp10)	7p, 9q, 11q	3p, 13	3q10, 7q10, 9q11, 11q11, 14p11, 16q24, 20q13, 22q13
Passage 262:			
Population a: 42-45, X, -Y, -3 or add (3) (p22), add (5) (q11), der (8) t (8;21) (p11;q11), add (9) (q12) or del (9) (?p23), i (10q), -10 or ?del (10) (q22), der (11) t (9;11) (q22;p15), der (14) t (3;14) (p11;p11), add (19) (q13), der (20) t (9;20) (q11;q13), +add (20) (q13), -21, der (22) t (5;22) (q13;q13) (cp11)	3q, 20	3p, 8p, 10p, -Y	3p22, 3p11, 5q11, 5q13, 8p11, 9q12, 9q11, 9q22, 10p11, 11p15, 14p11, 19q13, 20q11, 20q13, 21q11, 22q13
Sub-population a-1: 42-45, idem, del (1) (q11), +i(1q), -del(9), -add(20))(cp3)	1q,	9p	3p22, 3p11, 5q11, 5q13, 8p11, 9q12, 9q11, 9q22, 10p11, 11p15, 14p11, 19q13, 20q11, 20q13, 21q11, 22q13
Population b: 43-46, XY, add(3)(p12), add(9)(q12), -11, add(13)(p11), add(14)(p11), der(16)t(9;16)(q12;q24), der(20)t(9;20)(q11;q13), der(22)t(11;22)(q11;q13)(cp)	9q	3p, 11p	3p12, 9q11, 9q12, 11q11, 13p11, 14p11, 16q24, 20q13, 22q13
Sub-population b-1: 42-45, idem, -add(3), +der(3)t(3;7)(q10;p10), der(19)t(7;19)(q11;q11)(cp2)			3p12, 9q11, 9q16, 11q11, 13p11, 14p11, 16q24, 20q13, 22q13



**Fig. 4.** SSCP-PCR screening for the p53 gene mutation. Whole arrows indicate normal alleles; arrow heads indicate abnormal shifted bands. Lane 1 is P136, lanes 2, 3, 4 and HP are negative controls: lane 2 is normal oral epithelium (buccal), lane 3 is normal oral epithelium (gingival), lane 4 is normal lymph node and lane HP is human placenta DNA.

The typical wild type and mutation shifts of p53 are illustrated in Figure 4. A mutation band was seen as an extra band together with the wild type bands. The reason for the presence of wild-type bands may be the presence of a small number of normal cells, or heterogeneity in the tumor population. Mutation of p53 at exon 8 was detected in the cell line. No such mutation was present in the normal cells from which the immortalized line was developed.

The mutation detected from the passage 136 cell line was confirmed to be a point mutation at codon 273 of exon 8 representing a G to T transversion (Arg to Leu).

#### Tumorigenicity

The cultured cells were tested for tumorigenicity by subcutaneous injection of  $10^7$  cells per animal into nude mice, using both immortalized and normal cells. This was carried out at passage 217 and again at passage 305 of the IHGK cells. In neither case did either cell type result in development of tumor. The mice from the first study were still alive and healthy more than 6 months later.

#### Discussion

Our results demonstrate that HPV 16 E6/E7 gene immortalizes oral epithelial cells and leads to progressive chromosomal changes, but apparently does not result in tumorigenicity, as assessed by the nude mouse technique.

HPV infection alone does not necessarily lead to malignancy. In a similar study, five HPV immortalized cell lines with numerous deviant and altered chromosomes were non-tumorigenic in mice (31). All had cells with either homogeneously staining regions or double minute chromosomes, alterations associated with malignancy or drug resistance. Viral sequences were found on the abnormal chromosomes at junctions of chromosome translocations, at achromatic lesions and within homogeneously staining regions and duplicated chromosome segments (31). Cytogenetic analysis of eight HPV-immortalized human foreskin keratinocyte cell lines showed all were abnormal, containing a variety of numerical and structural aberrations (40). The viral DNA was integrated and all lines had extended lifespans, but were not tumorigenic. These cell lines were clonally and chromosomally stable over extended passages (40), in contrast to our cell line, which shows progressively accumulating chromosomal defects.

While HPV infection frequently immortalizes the host cells, it usually is not sufficient on its own to transform them to a malignant phenotype. Cells stated to be transformed by HPV have usually also been irradiated with UV light (41). However, prolonged passage in culture, or co-operation with activated *ras* oncogene, have been shown sufficient for full conversion to a malignant phenotype (26,42-45). R30 gingival fibroblasts from a patient taking phenytoin, which had a stable translocation between chromosomes 8 and 18 and expressed a higher steady state level of *c-myc*, were readily transformed with HPV-16, whereas normal gingival cells were not (46). Similarly, chemical carcinogens cause neoplastic conversion of HPV-immortalized oral cells, but not normal oral cells *in vitro*. When cells were treated with nitrosomethylurea (NMU) and TPA, only HPV-18 immortalized cells converted to a malignant phenotype, not normal cells (47). This may be due to the normal cells' ability to repair damaged DNA, an ability which is lost in the immortalized cells. Transient G1 arrest may be associated with enhanced levels of intranuclear wild type p53 protein (41). High risk HPV E6 protein binds to wild type p53 and increases degradation of p53 protein (13,48). Thus, unlike normal cells, immortalized cells readily convert to neoplastic cells because of their inability to arrest the cell cycle and repair DNA when challenged with genotoxic agents such as chemical carcinogens (41).

p53 mutations also are common in human cancer cells (49). Mutation of the p53 gene is found in human primary carcinomas of the cervix and cervical intraepithelial neoplasia containing HPV 33 infection (50). While our cells show no loss of chromosome 17p (the location of the p53 gene), nor its involvement in any of the chromosome rearrangements, we have demonstrated p53 gene mutation. This finding suggests that HPV 16 E6/E7 genes may be capable of inactivating the p53 gene not only by degrading p53 gene product, but also by causing mutation of the gene.

Among the numerous and progressive chromosomal changes monosomy 10p was clearly evident towards the late passages, i.e. passage 262. Monosomy 10p has been also reported by others with foreskin cells immortalized by HPV-18 and other human keratinocytes immortalized by HPV-16 (58,59). Pei *et al.* (58) suggested that chromosome 10p may be the site for a potential tumor suppressor gene. Many other chromosomal changes found in this cell line have been identified in one or more malignant human neoplasms or cultured immortalized cells (51). For example, trisomy 20 has been described in leukemia and epithelial bladder cancer, deletion of chromosome 8 is common in prostate cancer, deletion of chromosome 13 is common in retinoblastoma, and 9p monosomy indicates loss of the p16 tumor suppressor gene. This suggests that the mechanism of immortalization in these cells has much in common with the process of malignant transformation at other sites, in that multiple genetic changes, many at the same sites, are involved. By passage 290, these cells were growing at a much more increased rate and exhibiting morphology and chromosomal changes suggestive of transformation. However, they were, at this stage, still not tumorigenic in the nude mouse assay.

While studies of genetic and molecular markers in HPV-related cervical cancer have been widely reported, such studies in oral cancer are still at an early investigational phase. A recent study showed the presence of HPV types 16 and 18 in oral epithelial biopsies (52). HPV types 6 and 11 were found to be benign in the head and neck, as in the genital region.

Similarly, types 16 and 18 were found in malignant lesions, as in the genital region. Some HPVs, particularly type 16 variants, may be associated with ubiquitous asymptomatic oral infections (53) analogous to findings of HPV in uterine cervix of histologically normal women (54). Tobacco and alcohol are the main risk factors for head and neck cancers (8). Of 30 oral carcinomas studied with PCR, 27 were positive for oncogenic HPV type 16 or 18 DNA. Almost all had a history of tobacco use (8). HPV has only recently been identified as a risk factor for oral cancer, and its role is not yet sufficiently defined to classify it as a major factor, although it is present in up to 90% of head and neck cancers (8). Nor is it yet clear how HPV interacts with tobacco and alcohol in the development of malignancy; further data are required.

### Conclusions

Retroviral infection with HPV 16 E6/E7 genes successfully immortalized oral epithelial cells, which have now been in culture for almost 4 years and more than 350 passages. In contrast, normal cells can be maintained in culture for only five to seven passages. In morphology, the immortalized cells are more uniform and basaloid than the normal heterogeneous morphology and routinely show invasion of the matrix.

Flow cytometry shows that the IHGK cells have a significant increase in aneuploid population, and the chromosomal changes noted in these immortalized cells are numerous and demonstrate a progression from early to late passages, characteristics of transformed cell lines. Additionally, mutation of the p53 gene was detected. While these cells fail the classic test for malignancy, tumorigenicity in nude mice, they have many of the characteristics of transformed cells. It is possible that the nude mouse test is not adequate for testing malignancy in some types of cells, as shown by Chang *et al.* (55) for oral squamous cell carcinoma.

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# Suppression of various human tumor cell lines by a dominant negative H-ras mutant

C65

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A dominant negative H-ras mutant, N116Y, was transfected into a variety of human tumor cell lines. N116Y extremely inhibited the proliferation of A431 (vulva), PC3 (prostate), T24 (bladder), MCF7 (breast), NKPS and TMK1 (stomach) cancer cell lines. A431 and PC3 cells were particularly susceptible to N116Y. In order to examine the effects of N116Y on the neoplastic phenotypes, we transfected a less efficient N116Y expression vector into A431 cells. Almost all

clones survived after G418 selection. However, they did not retain the N116Y gene and only one clone faintly expressed N116Y. This N116Y-expressing clone had no tumorigenicity *in vivo*, and revealed deformed morphology and DNA fragmentation, suggesting that N116Y might have induced apoptotic cell death. Thus, N116Y may be applicable for gene therapy of a wide spectrum of human tumors.

## Introduction

Carcinogenesis is a multistep process. Accumulation of genetic changes, such as activation of oncogenes and loss of suppressor genes, promotes the stages of development of tumor cells [1]. Activation of *ras* oncogenes is one of the representative genetic changes which is frequently observed in various human tumors [2]. *Ras* p21s are essential signal transducers for cellular proliferation and differentiation, and acquire the transforming capacity by single point mutations. Moreover, overexpression of the normal H-ras proto-oncogene induces transformation of NIH3T3 cells [3, 4]. Inactivation of *ras* oncogenes, therefore, is a possible way to revert neoplastic phenotypes. Microinjection of a neutralizing anti *ras* p21 antibody caused morphological reversion in NIH3T3 cells transformed by *ras* and protein tyrosine kinase (PTK) oncogenes [5]. Disruption of the activated Ki-ras oncogene of human colon carcinoma cell lines raised the loss of anchorage independent cell growth and tumorigenicity *in vivo* [6]. We have already demonstrated that an H-ras mutant, N116Y, exhibited a dominant negative activity and suppressed the transformed phenotypes of NIH3T3 cells induced by overexpression of the H-ras proto-oncogene and several PTK oncogenes [7-9]. Since *ras* genes are highly conserved among mammals [10], N116Y can be a potent suppressor of human tumor cells.

In the present study, we transfected an efficient N116Y expression vector into various human tumor cell lines and found that N116Y could strongly inhibit the growth of these cells.

## Results

### Suppression of the various tumor cell lines by N116Y

To examine whether high expression of a dominant negative H-ras mutant, N116Y, affects tumor cell proliferation, we constructed an efficient N116Y expression vector, pZIP-N116Y, and transfected a variety of human tumor cell lines (A431, PC3, MCF7, NKPS and HeLa) and NIH3T3 cells transformed by a long terminal repeat (LTR)-linked rat H-ras proto-oncogene (18A) and *v-fes* (*fes-1*) (Table 1). Colony forming efficiencies determined by transfection of pZIPneoSV(X) showed that the lipofection procedure was available for all cell lines used in this experiment. Transfection of pZIP-N116Y completely inhibited the colony formation of A431, PC3, MCF7, NKPS and *fes-1*, and no cell survived after G418 selection. HeLa cells transfected with pZIP-N116Y formed many G418-resistant colonies. The majority of colonies, however, were tiny cell clusters and the cells which formed

Table 1 Numbers of G418-resistant colonies of various tumor cells

Cells	Tissue of origin	Number of colonies	
		pZIP-N116Y <sup>a</sup>	pZIPneoSV(X) <sup>a</sup>
<b>Human</b>			
A431	Vulva	0	309
MCF-7	Breast	0	124
PC-3	Prostate	0	776
NKPS	Stomach	0	216
HeLa	Cervix	9 <sup>b</sup>	130 <sup>b</sup>
<b>Mouse (NIH3T3)<sup>c</sup></b>			
18A	proto-H-ras	44	705
<i>fes-1</i>	<i>v-fes</i>	0	213

<sup>a</sup>5 µg of plasmid DNA was used in each experiment.

<sup>b</sup>Colonies with diameters of >1mm were counted.

<sup>c</sup>NIH3T3 cells transformed by the following oncogenes.

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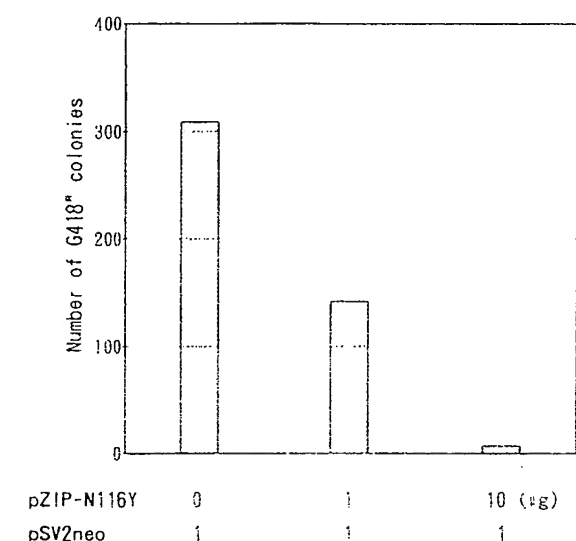


definite colonies ( $>1$  mm, on day 14) comprised fewer than 10% of colonies transfected with pZIPneoSV(X). The colony forming efficiency of 18A cells was greatly reduced by pZIP-N116Y and morphological reversion was induced at a high rate ( $>95\%$ ). These results suggest that pZIP-N116Y is a potent suppressor of various tumor cell lines.

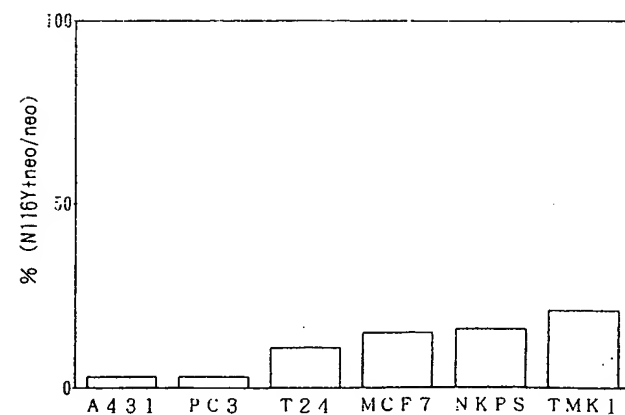
#### Cotransfection assays of N116Y H-ras mutant

In order to exclude the possibility that the suppressor activity of pZIP-N116Y depends on *neo* gene inactivation, we examined the colony forming ability of an A431 cell containing both pZIP-N116Y and pSV2neo by cotransfection of these plasmids at the rates of 10:1, 1:1 and 0:1 (Figure 1A). Total amounts of DNA were adjusted

A.



B.

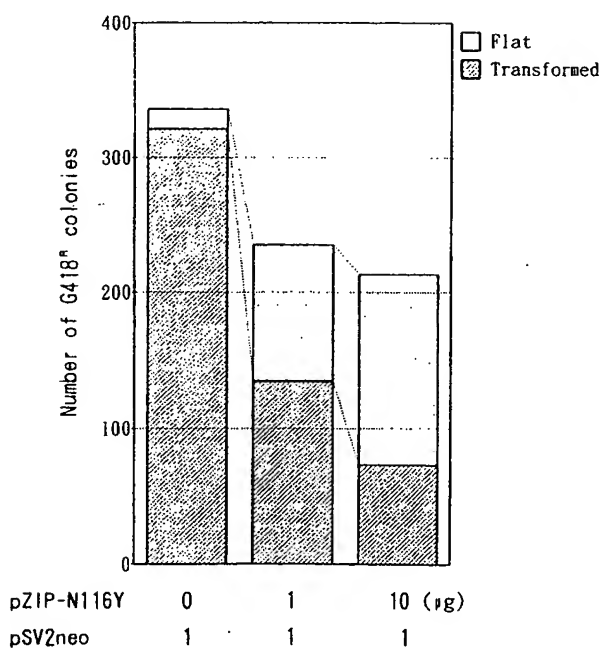


**Figure 1** Effects of pZIP-N116Y on the colony formation of human tumor cell lines. (A) A431 cells were cotransfected with pZIP-N116Y and pSV2neo at the ratios of 0:1, 1:1 or 10:1 ( $\mu$ g). The bars indicate the numbers of G418-resistant (G418<sup>r</sup>) colonies. Total amounts of transfected DNAs were adjusted to 11  $\mu$ g with sonicated salmon sperm DNA. (B) Cells were cotransfected with pZIP-N116Y and pSV2neo at the ratio of 10:1 ( $\mu$ g). The bars show the percentages of colony forming efficiencies relative to the control transfection (0:1). Averages of triplicated experiments are presented.

to 11  $\mu$ g with sonicated salmon sperm DNA. Compared with the control transfection (0:1) which generated more than 300 colonies, the number of colonies cotransfected with the equal amount of pZIP-N116Y and pSV2neo (1:1) was less than half. A higher rate of pZIP-N116Y (10:1) greatly inhibited colony formation, and the colony forming efficiency was only 3% of that of the control experiment. A reduction of colony forming efficiency by cotransfection means that most A431 cells introduced with pZIP-N116Y lost the growth ability.

We next analyzed the susceptibility of other human tumor cells to N116Y by cotransfection of pZIP-N116Y and pSV2neo at the ratio of 10:1 (Figure 1B). The numbers of G418-resistant colonies were 3% (PC3), 11% (T24), 14% (MCF7), 16% (NKPS) and 22% (TMK1) in comparison with the control transfection, indicating that N116Y suppressed the proliferation of these cell lines as well. Notably, a prostate cancer cell line, PC3, was as highly susceptible to N116Y as A431.

In order to confirm that growth suppression is associated with inhibition of cellular *ras* function, we investigated the relation between suppression of colony formation and morphological reversion induced by pZIP-N116Y. Since the *v-fes* oncogene requires *ras* activity for cellular transformation [5, 8], we cotransfected pZIP-N116Y and pSV2neo into *fes-1* cells (Figures 2 and 3). While a number of flat colonies appeared after cotransfection, colonies with transformed morphology were inversely correlated with the amount of pZIP-N116Y just like the G418-resistant colonies of A431 cells (Figure 1A). These results suggest that growth suppression is associated with inactivation of the cellular *ras* function by N116Y.



**Figure 2** Numbers and morphology of the G418-resistant (G418<sup>r</sup>) colonies of *fes-1* cells. The number of flat colonies exhibiting complete contact inhibition (stippled); the number of colonies with transformed morphology (hatched). Total amounts of transfected DNAs were adjusted to 11  $\mu$ g with sonicated salmon sperm DNA.

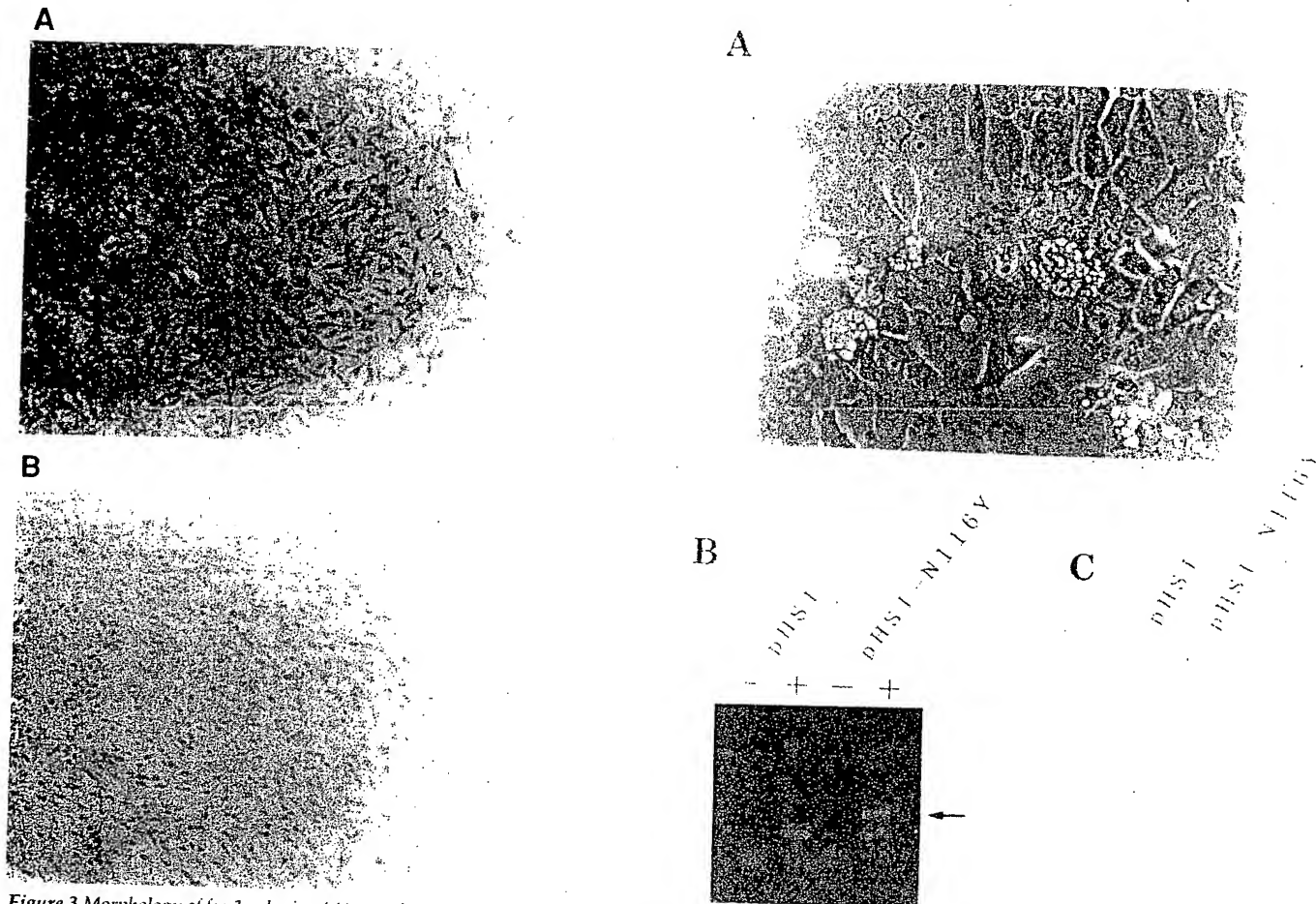


Figure 3 Morphology of *fes-1* colonies: (A) transformed-type; (B) flat-type. (Original magnification  $\times 100$ , Giemsa staining).

#### Effects of low expression of N116Y on A431 cells

To study the mechanism of suppression, we used a weak N116Y expression vector, pHSI-N116Y, which contains the human metallothioneine IIa promoter. Since this vector does not have a selection marker, we cotransfected with pHSI-N116Y and pSV2neo at the rate of 10:1 into A431 cells. After G418 selection, we isolated about 30 clones and used polymerase chain reaction (PCR) to screen for N116Y DNA. Unexpectedly, almost all clones had lost the N116Y gene. We finally obtained only one clone retaining N116Y. This clone contained a number of cells with distinctive morphologies: polynuclei; enlarged cells; monolayered growth; cellular fractionalization etc. (Figure 4A), together with a large number of disaggregated cells. Reverse transcriptase-PCR (RT-PCR) confirmed the expression of N116Y (Figure 4B), though Northern blot analysis was unable to detect the N116Y transcripts. For DNA analysis, we removed the disaggregated cells and harvested log-phase cells. These growing cells exhibited DNA fragmentation (Figure 4C). Moreover, this clone was not tumorigenic in nude mice (Table 2). These results suggest that a portion of this clone was in the process of apoptosis and N116Y affected the tumorigenicity of A431 cells at a low expression level.

Figure 4 (A) Morphology of an A431 clone, pHSI-N116Y, with low expression of N116Y. (B) Detection of N116Y transcripts by RT-PCR. Total RNA was incubated in the presence (+) or absence (-) of reverse transcriptase. The arrow indicates a specific band for N116Y. pHSI is a control A431 cell which was transfected with pHSI vector. (C) Analysis of DNA fragmentation. Soluble DNAs extracted from  $1 \times 10^6$  cells were labeled with [ $\alpha$ - $^{32}$ P]dCTP by Klenow fragment and were electrophoresed in 2% agarose gel.

#### Discussion

An *H-ras* mutant, N116Y, possesses the ability to suppress the transformed phenotypes induced by overexpression of the *H-ras* proto-oncogene [7], and confers a resistance to transformation by several protein tyrosine kinase oncogenes which act upstream to *ras* in

Table 2 Tumorigenicity of A431 clones in nude mice<sup>a</sup>

Cell	Tumors/mice	Latency <sup>b</sup> (days)
A431		
pHSI-N116Y	0/6	-
pHSII	6/6	<7

<sup>a</sup>Each mouse was inoculated subcutaneously with  $1 \times 10^6$  cells.

<sup>b</sup>Time required for the appearance of a palpable nodule at the site of inoculation.

the signaling pathways [8, 9]. In the present study, we found that transfection of an efficient N116Y expression vector, pZIP-N116Y, greatly reduced the proliferation of all human tumor cell lines examined. These results were unexpected, because Stacey *et al.* had reported that microinjection of a neutralizing anti-*ras* antibody did not block the proliferation of most human tumor cell lines excepting the cells containing a mutant *ras* gene [11]. Considering that the multiple alterations of genes regulating cell growth and differentiation are the main cause of cancer, transient inactivation of cellular *ras* by microinjection of an anti-*ras* antibody might not be sufficient to induce significant growth inhibition. We frequently observed that many cells transfected with pZIP-N116Y formed a small cell cluster at the early stage of G418 selection, but the majority of these cells died afterward. Continuous inactivation of cellular *ras*, therefore, may be important for suppressing the proliferation of tumor cells.

Cotransfection of pZIP-N116Y and pSV2neo showed that A431 and PC3 cells were particularly susceptible to N116Y. A431 is a squamous cell carcinoma line expressing a large amount of the epidermal growth factor receptor (EGFR) which is activated by an autocrine mechanism. Since *ras* proteins mediate the epidermal growth factor (EGF)-associated signals [12], N116Y probably prevents the autocrine cycle which may be required for proliferation of A431 cells. PC3 is an androgen-independent prostate cancer line which has no androgen receptors. High sensitivity to N116Y suggests that activation of *ras*-dependent signaling pathways in PC3 cells made up the lack of androgen receptor. N116Y might efficiently affect the cell lines which acquired growth advantages by enhancement of *ras*-associated signals.

A large reduction of the cellular *ras* function is probably cytotoxic to most cells. N116Y-induced growth suppression, however, may not necessarily depend on cytotoxicity of the N116Y products. An A431 clone with low N116Y expression had lost tumorigenicity *in vivo*, suggesting that N116Y is capable of affecting the neoplastic phenotypes of A431 cells. Moreover, this clone exhibited remarkable alteration of cellular morphology and DNA fragmentation which is characteristic of apoptotic cell death. Recent studies demonstrated that activated *H-ras* oncogenes could inhibit apoptosis of myeloid leukemia cells [13] and fibroblasts [14]. We have observed that N116Y induced apoptotic cell death on K562 human chronic myelogenous leukemia cells [15]. The *ras*-mediated signals, therefore, are probably associated with cell viability. High expression of N116Y might shut down these signaling pathways and induce apoptotic cell death.

The action of *ras* proteins is regulated by several guanine-nucleotide exchange factors and GTPase activating proteins [16, 17]. Down-regulation of the GTP/GDP exchange reaction or up-regulation of the GTP hydrolysis increases the inactive GDP-bound form of *ras* p21s. Hwang *et al.* reported that another *H-ras* mutant, N116I, which is biochemically identical to N116Y, formed a stable but catalytically inactive

complex with *Saccharomyces cerevisiae* SDC25 C-domain, a guanine-nucleotide exchange factor and inhibited *H-ras* p21 guanine-nucleotide exchange reaction [18]. N116Y may prevent the production of the GTP-bound form of the endogenous *ras* p21s by consuming the guanine-nucleotide exchange factors.

Intervention in the signaling pathways associated with cellular proliferation and neoplastic phenotypes is one of the approaches for cancer therapy [19]. In this report, we demonstrated that a dominant negative *H-ras* mutant, N116Y, is a potent suppressor of various human tumor cell lines. Introduction of N116Y *in vivo*, therefore, would be an effective approach for the treatment of tumors.

## Materials and methods

### Cell lines

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in 5% carbon dioxide at 37°C. Human tumor cell lines used in the present study were A431 (vulva), HeLa (cervix), PC3 (prostate), T24 (bladder), MCF7 (breast), TMK1 (stomach) and NKPS (stomach). Mouse transformed cell lines were 18A (NIH3T3 transformed by an LTR-linked rat c-Ha-*ras* proto-oncogene) and *fes-1* (*v-fes*).

### Plasmids

A dominant negative *H-ras* mutant, N116Y, was originally in the proviral DNA of Ha-MuSV which was cloned in the pSV2neo plasmid [20]. The 2.2-kb *Bam*HI-*Eco*RI fragment containing the N116Y mutant was inserted into the *Bam*HI site of pZIPneoSV(X) to construct pZIP-N116Y. Recombination of the *Bam*HI-*Eco*RI fragment of N116Y downstream of the human metallothioneine IIa promoter of pHSHI was used similarly to construct pHSHI-N116Y. The G418-resistant markers pSV2neo and pZIPneoSV(X) were used as the control.

### Transfection assays

Transfections were performed by a lipofection procedure as previously described [7]. In cotransfection assays, pZIP-N116Y and pSV2neo were mixed at 0:1, 1:1 and 10:1. The amount of DNA was adjusted to 11 µg with sonicated salmon sperm DNA. Transfected cells were divided 1:6–20 on the following day. The growth medium was changed to DMEM containing G418 (400 µg/ml) 4–6 h later. The G418 selection medium was changed on the next day and then every 3 or 4 days until G418-resistant colonies appeared. Numbers of G418-resistant colonies were counted after staining with Giemsa. For analysis of cell morphology, colonies were scrutinized under the microscope. Cells exhibiting a flat shape and a complete contact inhibition were scored as 'flat' colonies.

### Detection of DNA fragmentation

Log-phase cells ( $1 \times 10^7$ ) were harvested and washed once with phosphate buffered saline. These cells were resuspended in 0.6 ml lysis buffer (10 mM Tris-HCl, 10 mM EDTA and 0.2% Triton-X) and placed on ice for

10 min. The lysate was centrifuged at  $13\,000 \times g$  for 10 min. The supernatant was extracted four to five times with phenol-chloroform and precipitated with ethanol. The nucleic acids dissolved in distilled water were labeled with [ $\alpha$ - $^{32}$ P]-dCTP by Klenow fragment. The labeled DNAs were separated by electrophoresis in a 2% agarose gel. The agarose gel was then dried and autoradiographed on radiographic film.

#### Analysis of N116Y expression

To confirm the expression of N116Y, total RNA was analyzed by PCR after incubation with reverse transcriptase. The primers were 5'-GGTTTGGCAGCC-CCTGTAGAAG-3' (sense) and 5'-TACGCCCTGGTC-ATGTACGC-3' (antisense) to generate a 242-bp fragment. PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

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# **EXHIBIT 3**



# Molecular piracy: the viral link to carcinogenesis

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## Abstract

The vast majority of the human experience with viral infections is associated with acute symptoms, such as malaise, fever, chills, rhinitis and diarrhea. With this acute or lytic phase, the immune system mounts a response and eliminates the viral agent while acquiring antibodies to that specific viral subtype. With latent or chronic infections, the viral agent becomes incorporated into the human genome. Viral agents capable of integration into the host's genetic material are particularly dangerous and may commandeer the host's ability to regulate normal cell growth and proliferation. The oncogenic viruses may immortalize the host cell, and facilitate malignant transformation. Cell growth and proliferation may be enhanced by viral interference with tumor suppressor gene function (p53 and pRb). Viruses may act as vectors for mutated proto-oncogenes (oncogenes). Overexpression of these oncogenes in viral-infected cells interferes with normal cell function and allows unregulated cell growth and proliferation, which may lead to malignant transformation and tumour formation. Development of oral neoplasms, both benign and malignant, has been linked to several viruses. Epstein-Barr virus is associated with oral hairy leukoplakia, lymphoproliferative disease, lymphoepithelial carcinoma, B-cell lymphomas, and nasopharyngeal carcinoma. Human herpesvirus-8 has been implicated in all forms of Kaposi's sarcoma, primary effusion lymphomas, multiple myeloma, angioimmunoblastic lymphadenopathy, and Castleman's disease. Human herpesvirus-6 has been detected in lymphoproliferative disease, lymphomas, Hodgkin's disease, and oral squamous cell carcinoma. The role of human papillomavirus in benign (squamous papilloma, focal epithelial hyperplasia, condyloma acuminatum, verruca vulgaris), premalignant (oral epithelial dysplasia), and malignant (squamous cell carcinoma) neoplasms within the oral cavity is well recognized. Herpes simplex virus may participate as a cofactor in oral squamous cell carcinoma development by enhancing activation, amplification, and overexpression of pre-existing oncogenes within neoplastic tissues. Because of the integral role of viruses in malignant transformation of host cells, innovative antiviral therapy may prevent tumour development, involution neoplastic proliferations, or arrest malignant progression. © 1998 Elsevier Science Ltd. All rights reserved.

**Keywords:** Viruses; Cancer; HPV; HHV; EBV; KSHV; Proliferation markers; Oncogenes; Herpesvirus

## 1. Introduction

Viral infections are a common everyday occurrence and vary from a nuisance such as rhinitis and sinusitis (adenovirus, rhinovirus) and diarrhea (rotavirus, adenovirus), to infections which deplete the immune system and make the host susceptible to opportunistic infections (human immunodeficiency virus—HIV), and to latent infections which may result in the development of

a malignant process (Epstein-Barr virus—EBV) many years to decades later. Unfortunately, viruses which have tropism for the human host have the ability to induce or participate in malignant transformation via a number of mechanisms.

## 2. Pathogenesis of viral infections

Prior to discussing the viral link to carcinogenesis, the pathogenesis of viral infection needs to be reviewed briefly [1–4]. There are many sites of entry for viral agents and these include the gastrointestinal system, respiratory tract, skin and conjunctiva, urogenital tract,

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and blood via disruption in integrity of a vessel from insults such as trauma, surgery, and arthropod/mosquito bites. In order to infect a susceptible host, the virus must be able to survive in the environment. Many viruses which infect humans are delivered in body fluids and secretions (respiratory aerosols, saliva, blood, semen, vaginal secretions and serum/blood) which provide a protective environment for viral transmission to other human hosts. Specific receptors on cell surfaces allow attachment and adsorption of the virus envelope onto the host cell membrane. Once inside the cell (penetration), the virus uncoats and the viral genome enters the infected cell's nucleus and commandeers (molecular piracy) the host's genes, proteins and enzymes to synthesize viral DNA and RNA (transcription). Viral structural proteins necessary to produce the progeny viral particles are translated from the resultant RNA (translation and replication). These resulting structural proteins and newly synthesized viral DNA or RNA are packaged into a viral capsid (assembly) and enveloped by viral-directed glycoproteins produced in the host's cytoplasm (envelopment). The final product of viral infection of a host cell is the production of a mature enveloped virion which is released from the host cell and may go on to infect other host cells or other susceptible hosts. This phase is usually referred to as the lytic (acute) phase of viral infection because the infected host typically expresses cell surface markers, which alert the immune system to viral infection and a non-specific immune reaction results in lysis of the infected cell. Cellular and humoral immune responses will develop specific antibodies to the individual strain of virus that has infected the host; however, many viruses have numerous subtypes (HPV over 70 subtypes; adenovirus over 50 subtypes) and antibodies will not usually neutralize other subtypes of the same virus. This accounts for the multiple bouts of rhinitis and sinusitis that each of us encounter during our lifetime. Also during periods of immunosuppression or immunocompromise, induced by disease (systemic lupus erythematosus), drugs (steroids, chemotherapy) or other viruses, there may be an inadequate immune response to a previously encountered viral agent. Several viruses are capable of inducing acute (lytic) and/or chronic (latent) infection. In some hosts, viruses produce a minor, clinically undetectable acute infection, which may enter a latent phase (EBV infection). In other hosts, the acute infection may be quite severe (EBV-induced infectious mononucleosis) and require medical attention. The chronic latent phase of a viral infection differs from the acute lytic phase in that active viral replication does not occur. In latent infection, it is possible for the virus to become a permanent fixture of the host's nucleus and integrate its genome into the host cell. In some instances (EBV), the viral genome produces certain factors which allow for immortalization of the cell and pave the way

for the potential of malignant transformation. The preferred mode of infection for viruses is a lytic phase in which viral propagation occurs and allows infection of additional host cells and other hosts. Latent infection most often occurs in a non-reservoir host and is due to inefficient or ineffective commandeering of the host's genetic machinery for viral replication. However, with compromise or suppression of the immune system, reactivation of a latent infection to a lytic one is possible.

With retroviruses, infection of the host cell is similar except for the fact that these viruses are RNA rather than DNA viruses [1–4]. These viruses have a unique enzyme, reverse transcriptase, which allows DNA creation from the viral RNA. This DNA then becomes incorporated into the host's nuclear material and from there, RNA is transcribed and translated by the host for viral replication. The most commonly known retrovirus is the human immunodeficiency virus (HIV); however, there are numerous other retroviral agents which participate in human disease and development of tumors.

The molecular piracy of viruses extends past simply taking control of the synthetic function of the host cell. Viruses, oftentimes, integrate host genes within their own genome or create their own genes which are quite similar to those of the host [1–4]. Some of the gene products are beneficial in propagating a viral infection and replication of viral particles, as such viruses with 'host-like' genes and gene products may survive more readily than those that lack such features and is referred to as the natural selection process for survival. Many of these genes and gene products are associated with regulation of cell growth, proliferation and division. Others represent proto-oncogenes that, when inserted in incorrect areas within the host DNA by viral integration, result in the creation of oncogenes which may induce tumor formation (malignant transformation).

### 3. Viral influences on cell growth and proliferation

The vast majority of cells within the human body are in a stage of cell arrest; however, there are a certain percentage of cells which are progressing through the cell cycle [1–8]. Cells typically progress in an orderly process through a growth phase (G0/G1) to a synthetic phase when DNA is copied (S), to a second growth phase (G2) prior to cell division or mitosis (M) and back to a quiescent cell arrest phase (G0/G1) following cell division. This cell cycle is designed to have several checkpoints to evaluate cells with damaged or mutated DNA, which potentially could result in the development of a malignancy, if not repaired or eliminated from the cell population. Tumor suppressor gene products, such as p53 and pRb (retinoblastoma gene product), act within the cell cycle to allow for the repair of damaged or mutated DNA within individual cells or induce these



cells to undergo individual cell necrosis (programmed cell death or apoptosis). Tumor suppressor genes have become well known in the popular press during the past decade and medical research has identified several tumor suppressor genes which may be mutated and result in malignant proliferations [1–3,5–9]. The best known tumor suppressor gene products are p53 and pRb. Dysfunction in naturally occurring p53 (wild-type p53) or mutations in p53 are associated with many different types of malignancies. Similarly, dysfunction in wild-type pRb or mutations in pRb also result in various malignancies.

Several viruses influence the cell cycle by inhibiting the tumor suppressor ability of p53 and/or pRb [1–3,5–9]. In particular, the human papillomavirus produces gene products (E6 and E7) which interact with and bind wild-type p53 (E6) and wild-type pRb (E7) proteins [1–3,5,8,9]. This eliminates these protein's ability to redirect cells with damaged or mutated DNA toward a DNA repair pathway or toward individual cell necrosis (apoptosis). As a consequence, host cells with latent viral infection are allowed to proliferate without regulation by cellular repair or damaged cell elimination mechanisms. These viral-infected cells may continue to undergo additional mutations induced by other 'DNA-damaging' events, including tobacco, alcohol, carcinogens, toxins, ionizing radiation, prolonged ultraviolet light/sun exposure, chemical insult, other viral infections, which may result in the development of a malignant tumor (transformation process) [1–5]. Sv40 virus (T-antigen) and adenovirus (E1A and E1B) also have gene products which interfere with function of p53 and pRb [1–3]. Other viruses may not produce inhibitors to p53 and pRb directly, but instead form gene product proteins which enhance proliferation of cells.

#### 4. Viral-associated oncogenes

Oncogenes have been identified with several viruses, most prominently with RNA retroviruses [4–9]. Introduction of an oncogene into normal cells causes transformation into malignant cells, characterized by immortalization, unopposed cell proliferation, and invasive properties. In the human genome, there are between 50 000 to 100 000 genes and only about 50 genes have been identified as oncogenes [1–3,5,6,8]. Each of these oncogenes represent a mutated form of a proto-oncogene, which is already present in the normal host cell and plays an essential role in normal cell function. In the case where a virus has incorporated a mutated form of a proto-oncogene (oncogene) in its genome, the gene product of this oncogene will be overexpressed and result in the unregulated proliferation of the viral-infected cell. Retroviral (RNA) viruses and DNA viruses contain many oncogenes which cause lymphoma, myeloid

leukemias, sarcomas, and carcinomas. These viral-derived oncogenes produce transforming growth factors and receptors, signal activation factors, and nuclear transcription factors. Overexpression of these growth factors allows for interference with the regulation of normal cell function, growth and proliferation, and allows cells which overexpress these factors to escape cell proliferation regulation, leading to malignant transformation and tumor formation. Common oncogenes implicated in human oral malignancies include c-myc, H-ras, K-ras, ras/raf, c-erb-B-2, c-erb-B-1, TGF- $\alpha$ , Int-2, c-fgr, jun/fos, *prad-1*, and *bcl*.

#### 5. Prototypic viruses in oral malignancies

##### 5.1. Human herpesvirus 4 (HHV-4, Epstein–Barr virus)

Epstein–Barr virus (EBV) is a herpesvirus family member [4,5,8,10–15] that is associated with several neoplastic processes (Table 1). This virus has incorporated several factors which allow immortalization of epithelial cells and B lymphocytes. Several oncogenic gene products which are capable of inducing malignant tumor formation are induced by EBV latent infection (*bcl-2*, *bcl-10*, *c-fgr*, *jun/fos*) [4,5,8,10–14,16,17]. Certain EBV nuclear antigens have been shown to be oncogenic in their own right (EBNA1, EBNA2, LMP1) [4,5,8,10–14,16,17]. In addition, to these oncogenic features, another EBV nuclear antigen (EBNA-LP) interferes with wild type p53 and pRb function and results in dysregulation of the cell cycle.

Several EBV-induced neoplastic processes may be expressed in the oral cavity [4,5,8]. Oral hairy leukoplakia is a benign epithelial proliferation that occurs in immunocompromised (HIV-infected) or immunosuppressed (organ transplants, autoimmune disease) hosts. Lymphoproliferative disease is also seen in the oral cavity of transplant and autoimmune disease patients who are being managed with immunosuppressive drugs, such as corticosteroids, cyclosporine, azathioprine, methotrexate and cytoxan [4,5,8,11–14,17]. This disease process may be the precursor to the development of lymphoma. EBV is associated with several malignant processes which may present initially within the oral cavity. These include Burkitt's lymphoma, immunoblastic lymphoma, nasopharyngeal carcinoma, lymphoepithelial carcinoma of salivary glands, Hodgkin's disease, and leiomyosarcoma in pediatric HIV infection [4,5,8,10–17]. In HIV-positive individuals, there is a 20 to 60-fold increase in the prevalence of lymphoma [4,5,8,11–14,16,17]. Most of these are B cell lymphomas with EBV integration within the neoplastic cells. No doubt this may reflect the interactive effect of the transforming ability of both EBV and HIV tat protein (Table 1).



Table 1  
Human viruses with oncogenic potential

Virus	Tumor	Cofactors (putative)
Epstein-Barr virus	Burkitt's lymphoma immunoblastic lymphoma nasopharyngeal carcinoma Hodgkin's disease lymphoepithelial carcinoma leiomyosarcoma	malaria immunodeficiency nitrosamines, HLA type  pediatric HIV (tat protein)
HHV-8	Kaposi's sarcoma body cavity lymphoma Castleman's disease multiple myeloma	HIV (tat protein) HIV (tat protein) HIV (tat protein) HIV (tat protein)
HHV-6	oral squamous cell carcinoma Hodgkin's disease non-Hodgkin's lymphoma cervical carcinoma	HIV (tat protein) HIV (tat protein) HIV (tat protein) HIV (tat protein)
HPV	verrucous carcinoma squamous cell carcinoma koilocytic dysplasia (oral mucosa) epithelial dysplasia condyloma acuminatum focal epithelial hyperplasia squamous papilloma	alcohol, carcinogens smoking, HSV, HIV
Polyomavirus (BK, JC)	neural tumors insulinomas mesotheliomas	
Retroviruses (HTLV I) Retroviruses (HTLV II)	T-cell lymphoma, leukemia hairy cell leukemia	
Hepatitis B Hepatitis C	hepatocellular carcinoma hepatocellular carcinoma	afatoxin, alcohol, smoking afatoxin, alcohol, smoking

### 5.2. Human herpesvirus 8 (HHV-8, Kaposi's sarcoma-associated herpesvirus, KSHV)

Recently, HHV-8, a close relative of EBV, has been identified and is strongly linked with all forms of Kaposi's sarcoma, whether associated with HIV infection or not [4,10,11,18–20]. This new herpesvirus shares several features with EBV. It has oncogenic (transforming) ability via a viral encoded receptor (G-protein-coupled receptor), which induces cell proliferation mediated by vascular endothelial growth factor. In addition, ras/raf oncogenes are activated by a proliferation cascade initiated by HHV-8. Additional proliferation factors, which have been identified previously as oncogenic, are expressed by HHV-8 and these include *prad-1*, and *bcl-2*, both of which interfere with wild-type p53 and wild-type pRb regulation of cell proliferation.

HHV-8 has been identified in virtually all Kaposi's sarcomas, regardless of HIV status [4,10,18–20]. Kaposi's sarcoma occurs in 10 to 12% of HIV-infected individuals and in 15 to 20% of homosexual HIV-infected males [4,5,8]. Intraoral Kaposi's sarcoma is quite com-

mon in HIV-infected persons with 22% presenting with oral lesions and an additional 45% presenting with oral, skin and visceral involvement. In addition, HHV-8 has been associated with body cavity (primary effusion) lymphoma, multiple myeloma, angioimmunoblastic lymphadenopathy, and Castleman's disease, regardless of HIV infection status [4,10,11,18–21].

### 5.3. Human herpesvirus 6 (HHV-6)

Since the discovery of this herpes family virus in 1986, it has been detected in several lymphoid malignancies, including non-Hodgkin's lymphoma, Hodgkin's disease, cervical carcinoma, and oral squamous cell carcinoma [4,10,11,20–22]. In addition, a specific association between this virus and chronic lymphoproliferative disease appears to be possible. The strongest link between HHV-6 and possible malignant transformation may exist with Hodgkin's disease and oral squamous cell carcinoma. Transformation to malignancy may be facilitated by several factors induced by or expressed by HHV-6 infected cells. Tumor necrosis factor-alpha is

overexpressed and this appears to induce HIV replication which in turn increases HIV tat protein, a protein with oncogenic potential. In both immunocompetent and immunosuppressed hosts, HHV-6 produces an oncogenic protein (ORF-1) which binds to wild-type p53 and inhibits regulation of cell proliferation, which allows for the escape of HHV-6 infected host cells from cell growth regulation [4,19,21]. Further research is needed in order to determine if there is a relationship between HHV-6 and cancer or if it represents only a chance association.

#### 5.4. Human papillomavirus (HPV)

The papillomavirus is ubiquitous in higher vertebrate species, ranging from birds to humans. At the present time, over 77 types of human papillomavirus have been defined in humans based upon differences in DNA sequences [1–3,9]. HPV is known to produce several different squamous epithelial tumors [1–3,5,8,9]. The viruses have tropism for squamous cells and their full reproductive life cycle is only supported in these epithelial cells. In fact, HPV viral replication functions are limited to the most terminally differentiated squamous cells or keratinocytes. However, it is within the basal layer of squamous epithelium that viral DNA synthesis occurs and the incorporation of HPV genes into the basal cells ensures that a persistent lesion will develop. As indicated early, HPV produces two inhibitors of cell cycle regulation, namely E6 and E7 [1–3,5–9]. E6 binds wild-type p53 and E7 binds wild-type pRb. Inactivation of these tumor suppressor proteins leads to unregulated proliferation of the HPV-infected squamous cells and development of squamous epithelial tumors. In addition to these two inhibitors of cell cycle regulation, another HPV gene product (E5) stimulates growth factor activity, which enhances cell proliferation and may influence transformation to a malignant process [1–3,9]. The gene products E5, E6 and E7, are considered to be oncoproteins and are particularly evident with intermediate and high-risk HPV types (16, 18, 31, 33, 35, 39, 42) which are associated with the development of squamous cell carcinoma of the cervix and oral cavity [5,8,9].

Various HPV types have been associated with specific squamous epithelial lesions in the oral cavity [5,8,9]. Squamous papillomas are most commonly infected with HPV types 6 and 11. Verruca vulgaris, also known as the common wart, is associated with HPV types 1, 2, 4, 7 and 57. A sexually transmitted wart with potential for malignant transformation is the condyloma acuminatum. Fortunately, this type of proliferation occurs relatively infrequently in the oral cavity, but may be seen in HIV infection. Several intermediate and high-risk HPV types are identified with this venereal lesion and include HPV types 16, 18, 31, 33, and 35. In addition, HPV types 2, 6, and 11 may be detected in this

lesion. A benign condition which may mimic condyloma acuminatum is focal epithelial hyperplasia (Heck's disease). This is associated with low-risk HPV types 13 and 32 and malignant transformation has not been reported with this disease entity. A precursor to malignant transformation is HPV-associated oral epithelial dysplasia, which is also referred to as koilocytic dysplasia. This lesion is associated with intermediate and high-risk HPV types 16, 18, 31, 33, and 35.

Oral epithelial dysplasias, a precursor to squamous cell carcinoma, are infected with HPV [5,8,9] in up to 42% of cases. HPV types 2, 6, 11, 16, 18, 31, 33 and 35 have been detected in these dysplastic lesions. In fact, 75% of these lesions contain the high-risk HPV types 16 and 18. Oral verrucous carcinoma has a high detection rate (47%) for HPV integration into neoplastic cells. The viral types in verrucous carcinoma are 2, 6, 11, 16 and 18. Between one-third to one-half of oral squamous cell carcinoma have HPV detected. When HPV is present, 80% are infected with high-risk HPV type 16 and 18. Other HPV types isolated from oral squamous cell carcinoma include 2, 3, 13, 31/33/35, 52, and 57. It is important to note that cofactors, such as tobacco, alcohol, carcinogens, and other viral infections (HIV tat protein, herpes simplex virus), may participate along with HPV in the transformation process to oral cancer development.

#### 5.5. Herpes simplex virus (HSV)

A definitive oncogenic potential for herpes simplex virus has not been documented. In animal and in vitro tissue culture studies, HSV may participate in malignant transformation, chromosomal aberration/mutations and gene amplification in the presence of ultraviolet light and carcinogens (nitrosamine and other tobacco extracts) [4–8,11]. Perhaps, the role of HSV in carcinogenesis is in enhancing the activation, amplification and overexpression of pre-existing oncogenes within neoplastic tissue, such as c-erb-B-1 and c-myc. HSV binds and inactivates basic fibroblastic growth factor. It is the binding of this factor, which may activate certain oncogenes (c-myc). The presence of HSV gene products in oral carcinomas has been documented; however, this may simply represent reactivation of HSV infection due to the suppression of natural killer lymphocyte activity, which usually keeps HSV infection in check and in a latent phase [4,5,8]. Still HSV may participate, not as an oncogenic stimulus, but as a cofactor in squamous cell carcinoma development.

### 6. Future anti-viral therapy in cancer

It is quite obvious that viruses play an important role in the process of malignant transformation and, therefore, antiviral therapies may provide a means to prevent

or arrest the malignant progression [4,5,8,13]. Vaccines for various communicable viral disease have proven to be effective for eliminating certain diseases, such as mumps, measles, and poliomyelitis. While it is not possible to vaccinate individuals for all possible types and strains of viruses, it is feasible that people at high-risk for the development of a specific type of tumor may be vaccinated in the future for 'high-risk' virus strains and types in an effort to prevent or arrest a viral-associated tumor. Currently several anti-herpes viral agents are available; however, with identification of herpesvirus family members participating in neoplastic processes, it is likely that specific agents against EBV, HHV-6, and HHV-8 will be developed. Targeted gene therapy of tumor cells may also be on the horizon, in which the activity or products of oncogenes and inhibitors of cell regulation and proliferation may be neutralized by restoration of normal gene function. It is also possible that gene therapy may allow for integration of other retroviruses into tumor cells, while sparing normal cells. Infection of tumor cells with these retroviral agents could result in the destruction of neoplastic cells by a natural immune response or by antiviral agents directed against the retroviral-infected cells. Another avenue is to acquire natural killer lymphocytes from the host which have been sensitized to the antigens expressed by tumor cells. These sensitized lymphocytes could be grown in culture and returned to the host to seek out tumor cells and eliminate them by immunologic means. Finally, several inhibitors of various growth factor cascades are in the process of development and it may be possible to directly inject these within tumors. Perhaps, these inhibitors of growth factors will arrest tumor growth and/or partially involute the tumor in order to allow for adequate resection. With further sophistication of medical technology and the advancement of knowledge regarding tumor biology, innovative cancer treatment regimens based upon evolving biologic principles may be realized in the near future.

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# **EXHIBIT 4**

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**RECOMBINANT DNA ADVISORY COMMITTEE**

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**Minutes of Meeting**

**March 8, 2001**

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES  
Public Health Service  
National Institutes of Health

## CONTENTS

I.	Call to Order and Opening Remarks .....	2
II.	Minutes of the December 13 and 15, 2000 Meeting .....	2
A.	Committee Motion 1 .....	2
III.	Discussion of Human Gene Transfer Protocol #0101-443: Evaluation of the Safety and Effects of <i>ex vivo</i> Modification and Reinfusion of CD34+ Cells by an Antisense Construct Against HIV-1 in a Retroviral Vector .....	3
A.	Protocol Summary .....	3
B.	Written Comments From Preliminary Review .....	3
C.	RAC Discussion .....	4
D.	Investigator Response .....	4
E.	Public Comment .....	5
F.	RAC Recommendations .....	5
G.	Committee Motion 2 .....	5
IV.	Optimization of HIV-1 Vectors Containing an Anti-HIV Antisense Payload for Gene Transfer into HIV-Infected Individuals .....	5
A.	RAC Discussion .....	6
B.	Public Comment .....	6
V.	Discussion of Risk-Group Designation for Strain B of <i>E. coli</i> .....	6
A.	Committee Motion 3 .....	7
VI.	Proposed Action to Amend the <i>NIH Guidelines</i> Requirements for Serious Adverse Event Reporting: RAC Discussion and Vote .....	7
A.	Dr. Patterson .....	7
B.	Mr. Shipp .....	7
C.	Public Comment .....	8
1.	Abbey S. Meyers, National Organization for Rare Disorders .....	8
2.	Stephan E. Lawton, Biotechnology Industry Organization .....	8
3.	Rosemary Quigley, Council of Public Representatives .....	8
4.	Paul Gelsinger, Citizen .....	8
5.	W. French Anderson, University of Southern California/American Society of Gene Therapy .....	9
6.	Alan Milstein, Attorney .....	9
D.	RAC Discussion .....	9
E.	Committee Motion 4 .....	10
VII.	Discussion of Human Gene Transfer Protocol #0101-445: Clinical Protocol for Wild-Type p53 Gene Induction in Premalignancies of Squamous Epithelium of the Oral Cavity via an Adenoviral Vector .....	10
A.	Protocol Summary .....	10
B.	Written Comments From Preliminary Review .....	11
C.	RAC Discussion .....	12
D.	Investigator Response .....	12
E.	Public Comments .....	12
F.	RAC Recommendations .....	12
G.	Committee Motion 5 .....	12

VIII.	Proposed Plan for Addressing Issues Related to Institutional Biosafety Committees .....	13
A.	Mr. Shipp .....	13
B.	Dr. Dunn .....	13
C.	Dr. Braun .....	14
D.	RAC Discussion .....	14
E.	Public Comment .....	15
F.	Sense of the Committee .....	15
IX.	Data Management .....	15
A.	Amendments and Updates and Adverse Events .....	15
X.	Food and Drug Administration's Proposed Disclosure Rule .....	16
A.	RAC Comments .....	16
B.	Public Comments .....	17
1.	Dr. Braun .....	17
2.	Jo Ann Blake, Citizen .....	17
C.	Committee Motion 6 .....	17
XI.	Chair's Closing Remarks .....	17
XII.	Adjournment .....	17

Attachment I. Committee Roster

Attachment II. Attendees

Attachment III. Abbreviations and Acronyms

Note: The latest Human Gene Transfer Protocol List can be found at the Office of Biotechnology Activities' Web site at <<http://www4.od.nih.gov/oba/RDNA.htm>>.

**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES  
NATIONAL INSTITUTES OF HEALTH  
RECOMBINANT DNA ADVISORY COMMITTEE  
MINUTES OF MEETING<sup>1</sup>  
March 8, 2001**

The Recombinant DNA Advisory Committee (RAC) was convened for its 81st meeting at 8:30 a.m. on March 8, 2001, at the National Institutes of Health (NIH), Building 31, Sixth Floor, Conference Room 10, 9000 Rockville Pike, Bethesda, MD 20892. Dr. Claudia A. Mickelson (Chair) presided. In accordance with Public Law 92-463, the meeting was open to the public. The following individuals were present for all or part of the meeting:

**Committee Members**

C. Estuardo Aguilar-Cordova, Harvard Gene Therapy Initiative  
Dale G. Ando, Cell Genesys  
Xandra O. Breakefield, Massachusetts General Hospital  
Louise T. Chow, University of Alabama, Birmingham  
Theodore C. Friedmann, University of California, San Diego  
Jon W. Gordon, Mount Sinai School of Medicine  
Jay J. Greenblatt, National Cancer Institute, National Institutes of Health  
Eric T. Juengst, Case Western Reserve University  
Nancy M. P. King, University of North Carolina, Chapel Hill  
Sue L. Levi-Pearl, Tourette's Syndrome Association  
Ruth Macklin, Albert Einstein College of Medicine  
M. Louise Markert, Duke University Medical Center  
Claudia A. Mickelson, Massachusetts Institute of Technology

**Executive Secretary**

Amy P. Patterson, National Institutes of Health

**Ad Hoc/Speakers**

Andrew George Braun, Harvard Medical School  
Boro Dropulic, VIRxSYS  
Cynthia Dunn, Clinical Research Institute  
John J. Fung, University of Pittsburgh  
Carter Van Waes, National Institute on Deafness and Other Communication Disorders, NIH

**Nonvoting/Agency Representatives**

Kristina C. Borrer, Office for Human Research Protections, Department of Health and Human Services  
Philip Noguchi, U.S. Food and Drug Administration

**National Institutes of Health Staff Members**

Sarah Carr, OD  
Janita Coen, NHLBI  
J.R. Dixon, OD  
Kelly Fennington, OD

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<sup>1</sup> The Recombinant DNA Advisory Committee is advisory to the National Institutes of Health (NIH), and its recommendations should not be considered as final or accepted. The Office of Biotechnology Activities should be consulted for NIH policy on specific issues.



Joseph F. Gallelli, CC  
Robert Jambou, OD  
Kathryn R. Lesh, OD  
Barbara McDonald, OD  
Cheryl McDonald, OD  
Marina O'Reilly, OD  
Alexander Rakowsky, OD  
Gene Rosenthal, OD  
Thomas Shih, OD  
Allan Shipp, OD  
Sonia I. Skarlatos, NHLBI  
Lana Skirboll, OD

**Others**

Approximately 45 individuals attended this 1-day RAC meeting. A list of attendees appears in Attachment II.

**I. Call to Order and Opening Remarks/Dr. Mickelson**

Dr. Mickelson, RAC Chair, called the meeting to order at 8:30 a.m. on March 8, 2001. Notice of this meeting under the *NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines)* was published in the *Federal Register* on February 23, 2001 (66 FR 11305). The agenda included reviews of two gene transfer protocols, data management, the proposed action to amend the *NIH Guidelines* requirements for reporting and analysis of serious adverse events, a proposed plan for addressing issues related to the roles and responsibilities of Institutional Biosafety Committees, a lentiviral vector system under development for use in clinical trials, risk-containment practices for strain B of the common bacterium *Escherichia coli* (*E. coli*) frequently used for large-scale production processes, and the U.S. Food and Drug Administration's (FDA) proposed disclosure rule, "Availability for Public Disclosure and Submission to FDA for Public Disclosure of Certain Data and Information Related to Human Gene Therapy or Xenotransplantation."

Following a review of conflict-of-interest rules, Dr. Mickelson offered a brief summary of the March 7, 2001 Fourth National Gene Transfer Safety Symposium: Safety Considerations in the Use of AAV Vectors in Gene Transfer Clinical Trials. Several RAC members noted that this symposium was an example of how regulatory and review bodies can respond quickly to an ongoing concern within the scientific community. The Office of Biotechnology Activities (OBA) was congratulated for organizing the symposium and putting together an effective program, and Mark S. Sands, Ph.D., Washington University School of Medicine, was lauded for generating awareness among the scientific community and the public of the issues raised by his preclinical research results.

**II. Minutes of the December 13 and 15, 2000 Meeting/Drs. Gordon and Juengst**

Dr. Gordon noted that a few technical words were misspelled, and he provided a copy of the minutes that included those corrections.

**A. Committee Motion 1**

As moved by Dr. Gordon and seconded by Dr. Markert and with the understanding that any misspellings will be corrected, the RAC unanimously approved the December 13 and 15, 2000 minutes by a vote of 12 in favor, 0 opposed, and 0 abstentions.

**III. Discussion of Human Gene Transfer Protocol #0101-443: Evaluation of the Safety and Effects of *ex vivo* Modification and Reinfusion of CD34+ Cells by an Antisense Construct Against HIV-1 in a Retroviral Vector**

Principal Investigator: Jeffrey C. Laurence, M.D., Weill Medical College, Cornell University  
Other Investigators: Marcus A. Conant, M.D., Dermatology/HIV Consultant;  
Dean L. Engelhardt, Ph.D., Enzo Therapeutics;  
Barbara E. Thalenfeld, Ph.D., Enzo Therapeutics  
Sponsor: Enzo Therapeutics, Inc.  
RAC Reviewers: Dr. Aguilar-Cordova, Ms. King, and Drs. Markert and Mickelson  
Ad Hoc Reviewer: John J. Fung, M.D., Ph.D., University of Pittsburgh

**A. Protocol Summary**

Investigators have demonstrated that the growth of HIV-1 can be blocked by the use of antisense genes. Three independent antisense sequences directed against 2 HIV-1 functional regions, *tar* and *tat/rev*, have been embedded into separate cloned human U1 RNA genes. This triple U1/HIV-1 antisense cassette was incorporated into a Moloney murine leukemia virus derived vector (HGTV43) used to transduce CD34+ cells. Preclinical data suggested that the presence of the anti-HIV-1 genetic antisense RNA in CD4+ cells would be sufficient to manage HIV-1 levels in infected subjects.

A phase I clinical trial was initiated in which peripheral blood stem cells (PBSC) from HIV-1 infected research participants were transduced with HGTV43 and reinfused. Results from the clinical protocol demonstrate long term (6-12 months) survival of antisense RNA in a low number of bone marrow stem cells as well as in the peripheral blood mononuclear cells (PBMC) and CD4+ fraction. Since this low number of transduced PBMC has remained constant over a number of months, these data support the conclusion that stable engraftment of some of the antisense RNA-producing PBSC has occurred. Finally, there is no evidence that multiple infusions led to increased levels of engraftment.

This protocol is a continuation of the trial reported above. The investigators propose to increase the number of CD4+ cells producing anti-HIV-1 genetic antisense RNA. The investigators propose to isolate a population of PBSC from HIV-1 positive participants previously treated with G-CSF. After this isolation, the participants will receive a treatment of immune conditioning using mycophenolate mofetil (MMF). The PBSC will be transduced with HGTV43. After the transduction is complete, the participants will be irradiated in an outpatient procedure (600cGy, TBI), and the transduced cells containing the antisense genes will be re-infused into the HIV-1 participant. The end points of this study are the safety of the procedure and the extent of engraftment and proliferation of the engineered cell population. The study will enroll up to 6 participants.

**B. Written Comments From Preliminary Review**

Seven RAC members recommended that the protocol warranted public discussion. Ms. King and Drs. Markert and Mickelson submitted written reviews, as did *ad hoc* reviewer Dr. Fung, to which the investigators responded in writing and during this meeting.

Dr. Aguilar-Cordova raised a concern about the potential for high risk to the participants if radiation increased viral load simultaneous with reducing the immune response. He also asked which chemotherapeutic agent would be used and its effect on HIV Long Terminal Repeat (LTR) expression. Another issue of concern was the stability of the integrated vector. The vector contains three repeat sequences which increase the potential for recombination. He requested that the investigators provide more information about the packaging cell line.

In order to calculate the risk/benefit ratio, Ms. King requested more information on the effects of total body irradiation (TBI): potential for improved engraftment vs. immune suppression. Regarding the informed consent document, Ms. King noted that the potential benefit to participants was overstated, the statement of the risk of bone marrow suppression should be emphasized, an autopsy request should be included, an

appropriate financial disclosure statement should be added, and the document should be rewritten in second person.

Dr. Markert asked about the effect of irradiation on existing peripheral T cells and the thymus since these are sources of T-cell renewal in adults. If the thymus is damaged prior to or during irradiation, the participant would be unable to regenerate functional T cells. She recommended that only participants with proper thymic function should be enrolled and that, if the first two participants' subsequent test results show the thymus has been damaged such that T cells cannot be made, the study should be halted. She asked that the vector facility be properly audited to ensure that Good Manufacturing Procedures are followed, that procedures be performed on participants in a clinic rather than a physician's office, and that a Data and Safety Monitoring Board (DSMB) be established.

Dr. Mickelson focused on concerns about whether the use of the proposed conditioning/ablation treatments would significantly increase the risks associated with trial participation for this patient population. She also questioned whether using TBI would increase the risk of neoplasia, or the rate of appearance of HIV variants, thus affecting the efficacy of concurrent drug therapies. Because of these possible effects, she asked why TBI was selected rather than high-dose chemotherapy. She also questioned the low efficiency of transduction of CD34+ cells and which differentiated cell types might express the antisense RNA after engraftment.

Dr. Fung expressed concerns about the lack of information in the preliminary data regarding multiple-dose subjects, the effect of TBI on HIV replication, the use of immunosuppressive agents in an autotransplant, and the use of human serum for the isolation of CD34+ cells.

#### **C. RAC Discussion**

Several issues were raised by RAC members during discussion in addition to those expressed by the initial reviewers:

- Dr. Markert asked why the researchers used fetal calf serum in this protocol.
- Dr. Friedmann expressed concern that the vector construct may be prone to genetic rearrangements.
- Ms. Levi-Pearl commented that the informed consent document did not disclose information regarding whether the investigators have financial interests with the sponsor.
- Dr. Markert suggested that investigators use an immunoscope prior to and after the procedure to provide useful immunologic information about thymic function. The DSMB could use these comparative data to keep track of subjects' immune status and to decide whether to halt the trial.

#### **D. Investigator Response**

Regarding the concern about administering product in a physician's office, Dr. Conant responded that his office is set up to respond to severe hypotension and other immediate reactions, and provides both nursing staff and appropriate equipment. He agreed with Dr. Markert's suggestion of starting the trial with two subjects and then asking a DSMB with immunology expertise to review those results before proceeding. As to testing subjects for thymic function, Dr. Conant expressed his belief that only a small percentage of patients would be excluded as a result of thymic dysfunction, but agreed to implement that test. In response to Dr. Mickelson's question about using radiation therapy instead of chemotherapy, he stated that chemotherapy would be more detrimental than the low-dose radiation therapy proposed for this trial.

Dr. Laurence stated that the level of radiation proposed is standard treatment at New York Hospital's transplant unit and has been approved for treating cancer patients who are HIV positive. In response to Dr. Fung's questions about the proposed immunosuppressive regimen, Dr. Laurence responded that it is a mild, immune-conditioning regimen and that MMF appears to be an effective anti-HIV agent that

synergizes with other anti-HIV drugs. Dr. Conant noted mycophenolic acid is also useful in arresting differentiation of the transduced CD34+ cells following readministration to the participant. Dr. Laurence also indicated that fetal calf serum would be replaced with human serum to avoid possible antibody formation.

In response to Dr. Aguilar-Cordova's suggestion to use Southern blot analysis on target clones to check for rearranged vectors in the transduced cells, Dr. Engelhardt stated that the investigators have assayed by amplification of the insert rather than performing Southern blots on transduced cells.

**E. Public Comment**

No public comments were offered.

**F. RAC Recommendations**

Dr. Mickelson summarized the RAC recommendations as follows:

- A DSMB should be used to review the thymic function data and other safety information to determine whether the study should continue or what the next steps should be. At least one immunologist should be involved as immunologic adverse events may well occur. For research subject safety, this expert input on the review of safety data and protocol design is important.
- Southern Blot analysis should be conducted to assess vector construct stability in both the vector producing cells and the transduced cells.
- With regard to the informed consent document, the partial ablation radiation and pre-conditioning regimen should be clarified and this explanation should be moved forward to a more prominent position in the document. Also, the informed consent document should disclose whether the investigators have financial interests in Enzo Therapeutics, Inc.
- In order to diminish the host immune rejection response, xenoproteins such as fetal calf serum should be replaced with human or autologous proteins where possible.

**G. Committee Motion 2**

It was moved by Dr. Aguilar-Cordova and seconded by Dr. Markert that the recommendations expressed the views of the RAC and, following review by the RAC members and ad hoc reviewers, would be reiterated in a letter to the investigators and sponsor. The vote was 13 in favor, 0 opposed, and 0 abstentions.

**IV. Optimization of HIV-1 Vectors Containing an Anti-HIV Antisense Payload for Gene Transfer into HIV-Infected Individuals/Boro Dropulic, Ph.D., VIRxSYS**

Dr. Dropulic presented a lentiviral vector, VRX496, being developed for use in *ex vivo* gene transfer into HIV patients. The vector is derived from HIV-1, and does not encode any viral proteins. Expression of an antisense RNA to HIV-1 envelope is controlled by the HIV LTR, limiting expression only to HIV infected cells also expressing Tat and Rev. The clinical goal would be to interfere with wild type (wt)-HIV *in vivo* to decrease the viral load set point and to increase CD4 T-cell survival in order to postpone the development of acquired immune deficiency syndrome. Because the vector consists of only HIV-1 sequence, it may have safety advantages since it would introduce no new sequences into possible recombinants between the vector and wt-HIV-1; therefore, any replication-competent recombinants generated would not have the potential to be more pathogenic than wt-HIV.

In vitro results were presented showing the efficiency of human CD4 cell transduction and inhibition of HIV replication in challenged transduced cells. Biodistribution studies were performed in a mouse NOD/SCID model injected with transduced human T cells.

Dr. Dropulic also outlined the design of the clinical trial that is expected to be mounted. It would involve *ex vivo* transduction of CD4 T cells isolated from the research participant. Before re-administration, the cell product would be assayed for the presence of helper RNA or DNA. Research participants in this incremental dose-escalation trial would be monitored for differential viral load, CD4 count, and replication competent retrovirus (RCR).

#### **A. RAC Discussion**

Dr. Aguilar-Cordova asked why a vector that did not mobilize well was being pursued since mobilization should amplify the inhibitory effect while a vector without that capability would not have any advantage over MLV based retroviral vectors. Dr. Dropulic agreed that mobilizing vectors would have increased efficacy, but the vector was chosen for its safety features. He suggested that an HIV-1 derived vector would be more effective because the vector HIV RNA would track intra-cellularly to the sites of wt-HIV-1.

Dr. Mickelson and Dr. Friedmann asked about potential problems with toxicity or immune responses to the vector pseudotyped with the Vesicular Stomatitis Virus G (VSVG) envelope. Dr. Ando pointed out that because of the potential for recombination, the choice of envelope is not trivial and assays for RCR are limited in their sensitivity. Dr. Dropulic described the lot release criteria involving polymerase chain reaction (PCR) detection of helper RNA or DNA sequence and cell assays to look for any potential replication-competent virus.

Dr. Markert suggested that participants should be followed with an immunoscope to test for immune diversity. Dr. Dropulic responded that in the animal studies for which preparations are currently under way, immunoscopic analysis of the cells will be performed and cells will be tested by fluorescence-activated cell sorter for various receptors. Dr. Markert indicated that the new cytokine assays might result in allergies.

#### **B. Public Comment**

No public comments were offered.

#### **V. Discussion of Risk-Group Designation for Strain B of *E. coli*/Drs. Ando and Mickelson**

Dr. Ando explained that the University of Florida requested a definition of the risk-group classification for *E. coli* B strain be developed. Strain B is widely used in industry for fermentation and large-scale manufacturing of proteins because of the increased stability of cloned sequences compared with that of *E. coli* K-12.

Dr. Mickelson suggested that all *E. coli* strains could be placed into Risk Group 1 (RG1), nonpathogenic organisms, provided they lack virulence genes, contain deletions in metabolic genes so they are dependent on laboratory media, and do not make any known toxins. She explained that certain *E. coli* strains, such as K-12, are exempt from the *NIH Guidelines* because they meet a fourth criterion: inability to colonize the human gut. Rather than making decisions on a strain-by-strain basis, she suggested the generation of a general statement outlining the characteristics required for *E. coli* strains to be designated RG1 under the *NIH Guidelines*. Dr. Patterson indicated that a strawman proposal for this had been developed which could be put forward as a proposed action.

In the interim, a letter will be drafted in reply to the University of Florida's request that its strain of *E. coli* be considered RG1, as long as it does not contain toxins or virulence factors and there is metabolic dependence on laboratory media. Drs. Ando and Mickelson will work on the wording of the letter and distribute it to RAC members for review before sending.

Proposed language to amend the *NIH Guidelines* will be brought to the RAC at its next meeting and will then be published in the *Federal Register* for a public comment period. Dr. Mickelson offered the following general phrasing of the amendment language: If a strain can be shown not to produce any of the known bacterial toxins, does not contain any of the known major virulent factors for *E. coli*, and it carries

deletions in the metabolic genes that make it dependent on laboratory media, then those strains should be considered as Risk Group 1 *E. coli* for both large-scale and laboratory work.

**A. Committee Motion 3**

It was moved by Dr. Gordon and seconded by Dr. Markert that the strain of *E. coli* proposed by the University of Florida be considered Risk Group 1 and that draft language be developed to amend the *NIH Guidelines*. The vote was 12 in favor, 0 opposed, and 1 abstention.

**VI. Proposed Action To Amend the *NIH Guidelines* Requirements for Serious Adverse Event Reporting (SAER): RAC Discussion and Vote/Dr. Macklin**

Dr. Macklin called on Dr. Patterson and Mr. Allan Shipp, OBA.

**A. Dr. Patterson**

Dr. Patterson presented an overview of the Proposed Action that would amend the *NIH Guidelines* to enhance the reporting of safety information, its assessment, and its communication to the scientific community and the public. There are four elements to the proposal: (1) harmonization of the scope and timing of SAER to create one set of reporting criteria to both the NIH and FDA; (2) public access to safety information that will not be considered trade secret; (3) protection of research participant privacy in SAER; and (4) establishment of a national data assessment board. The Proposed Action establishes one set of reporting criteria for researchers to follow for both NIH and FDA and will provide enhanced and systematic analysis of safety data across all trials that will be presented publicly to inform about the design and conduct of ongoing and future clinical trials.

The proposed Gene Transfer Safety Assessment Board (GTSAB) would function as a mechanism for collecting, analyzing, and publicly reporting safety information across all trials. As such, it would facilitate early recognition of trends; report findings, conclusions, and aggregated trend analyses for public discussion at RAC meetings; and inform research participants, clinical investigators, basic scientists, Institutional Review Boards (IRBs) and Institutional Biosafety Committees (IBCs), and the public. The GTSAB would operate in an analytic and advisory capacity and would not supersede or replace the responsibilities of FDA or local review bodies in the day-to-day review of, and real-time response to, safety information. Approximately 15 members would make up this new Board, with outside experts in relevant fields constituting the majority of its membership; other members would include two RAC members, and NIH and FDA members. The board would meet quarterly in closed sessions prior to RAC meetings and provide reports to the RAC as well as publish periodic summary reports and cumulative trend analyses.

Dr. Patterson also reported on the status of the development of a national database for gene transfer clinical research. Using a controlled reporting vocabulary, this relational database will include product descriptors, elements of clinical trial design, and safety and toxicity data. It will be query capable and Web based. As an analytic tool for FDA, NIH, and advisory boards, this database will facilitate the evaluation and analysis of safety information from all gene transfer clinical trials. Reports from the database will inform diverse user groups such as IRBs, IBCs, local DSMBs, investigators, research participants, and the general public. Currently, the basic data structure and software design are nearing completion, and a draft common adverse event (AE) reporting form acceptable to both NIH and FDA staffs has been completed. The next steps include obtaining input from other user groups to finalize system software and training investigators and sponsors in the use of controlled vocabularies.

**B. Mr. Shipp**

Mr. Shipp summarized the public comments on the Proposed Action for SAER. Thirty-four sets of comments were received: two from professional associations, one from a scientific association, three from industry associations, six from patient groups and associations, three from academic officials, four from pharmaceutical and biotechnology companies, and the remainder from individuals. According to those comments, the prohibition of submission of individually identifiable patient data was supported

universally. Public access was also generally favored although there were differing views about the definition of confidential commercial information. Regarding the timing and scope of SAER, the majority of comments favored harmonization; however, industry and the National Hemophilia Foundation believe that no raw SAEs should be reported to the RAC, but rather that the RAC should rely on FDA for that information. A majority of respondents stated their belief that the RAC and the proposed GTSAB can serve a unique and necessary role in the public dissemination of safety and ethical information regarding gene transfer research (GTR) given that FDA is bound by confidentiality restrictions.

**C. Public Comment**

**1. Abbey S. Meyers, National Organization for Rare Disorders (former RAC member)**

Ms. Meyers described one role of the RAC as informing a public fearful of gene transfer research. In the wake of the Jesse Gelsinger tragedy and problems with genetically modified foods, public trust is eroding. The proposal is necessary to prevent the rejection of gene therapy as is happening with agricultural biotechnology. Gene therapy will fail if the public withdraws its trust in research, the researchers and the government's ability to protect the people. She urged the adoption of the proposed action and suggested that industry needs to realize that gene therapy is not just about money; it is about lives.

**2. Stephan E. Lawton, Biotechnology Industry Organization (BIO)**

Mr. Lawton began with assurances that BIO supports the reporting and analysis of safety data. However, they interpret the proposed action as, for the first time in the history of DHHS, compelling the submission and revelation of confidential commercial information to the public. This would make information accessible to competitors and could constitute a significant risk to smaller biotechnology companies, particularly in their ability to attract venture capital. He requested an invitation to work with the RAC/NIH on the proposed action prior to its approval.

Dr. Mickelson requested clarification of BIO's position in light of the fact that this same type of information has been requested, released, and discussed by the RAC for a decade. Mr. Lawton replied that some of the information requested in Appendix M could be of advantage to competitors; therefore, they objected to not being able to label it trade secret. Dr. Patterson reiterated that the proposal refers to a set of data that has already been requested for 10 years with the provision that it not include confidential commercial information. If it is marked as such, decisions will be made on a case-by-case basis allowing for dialogue with the investigator. She emphasized the need to be true to the spirit of the proposal to which Mr. Lawton requested again to work with NIH on the letter of the proposal.

**3. Rosemary Quigley, Council of Public Representatives (COPR)**

By speakerphone, Ms. Quigley expressed her concerns about the adequacy of research subject protection and the need for patient access to information necessary for truly informed consent. COPR strongly supports adoption of the proposed action as drafted. In order to protect participants and advance the nascent field of GTR, she stressed the importance of reporting all adverse events when there is any possibility of association with the gene transfer product. The creation of the GTSAB was endorsed as a necessary complement to the reported raw data that may become available under the FDA proposal. She stated her appreciation that in addition to the RAC review of protocols, NIH would now take the responsibility for the informed dissemination of SAE information. Regarding the BIO statement, COPR views public disclosure of SAEs and discussion of the analyzed data as assistance, not a hindrance, to industry.

**4. Paul Gelsinger, Citizen**

Mr. Gelsinger stated his belief that a major reason for his son's death in a gene transfer clinical trial was the financial pressure upon medical research that caused money to become more important than the welfare of clinical trial participants. He urged researchers to properly report all AEs and to allow NIH to

discuss and review events related to GTR, and that FDA be allowed to release more information to the public. He stated that this proposed action is an appropriate step toward getting GTR on the correct path.

**5. W. French Anderson, University of Southern California/American Society of Gene Therapy (ASGT)**

Speaking on behalf of ASGT, Dr. Anderson stated that ASGT is very much in favor of the Proposed Action and the proposal to allow FDA to be more open regarding SAE reports. Although he expressed support for the spirit of these proposals, he was concerned that, in the enthusiasm to implement them, certain aspects could cause problems, so he suggested working with BIO and other individuals.

**6. Alan Milstein, Attorney**

Mr. Milstein queried the RAC about the meaning of Mr. Lawton's statement that "we can work out" the concerns of the biotechnology industry. He was apprehensive that negotiations might result in the removal of the requirement for public disclosure of SAEs.

**D. RAC Discussion**

Issues discussed included the following:

- Dr. Skirboll clarified the following points: SAEs would be submitted to NIH in a manner harmonized with FDA submission. The GTSAB analysis would not occur in public, but the reports generated would be publicly discussed by the RAC. As with any raw data that come to NIH, this data would also be publicly accessible if requested under the Freedom of Information Act (FOIA). Should there be any substantive changes to the proposal, it would have to be brought back to the RAC for another vote. Because the *NIH Guidelines* can be amended if necessary, further changes may be made should the FDA public disclosure rule become regulation.
- Ms. King reminded everyone present about the language in Appendix M of the *NIH Guidelines* about proposals not containing trade secrets or confidential commercial information; she reiterated that nothing in the Proposed Action changes that language, which has been in effect for about 10 years, and she suggested that RAC discussion center on aspects of the Proposed Action other than the wording found in Appendix M.
- Dr. Markert stated that GTR is not particularly high risk in relation to other research; however, the Proposed Action is necessary to allay the public perception of it as such. Another misconception is that the GTSAB would be reviewing individual SAEs. In actuality, it would review data in the aggregate. Individual review of SAEs could continue to be the responsibility of the local DSMBs. Dr. Markert also noted that a database of raw SAE information on the Web that can be accessed by anyone may be a disservice to the public. Dr. Patterson and Dr. Greenblatt explained that while analysis of the data would be available, the raw data and the preanalysis would be sheltered behind a firewall. Raw data would be available only through FOIA requests to OBA.
- Dr. Jay P. Siegel, FDA, explained that FDA does assess AE in a manner similar to that proposed for the GTSAB, but FDA recognizes that this potential duplication of effort is currently necessary due to the restrictions on public disclosure by FDA. In the event that FDA's disclosure rules are loosened, it would be appropriate to review the coordination of FDA and NIH efforts. Dr. Siegel described some of the issues related to the review of safety data, particularly noting that the aggregate assessment of safety data is a complex process. He further noted that the GTSAB will likely review a database that is somewhat different from the one FDA reviews because of disclosure issues. Dr. Siegel reiterated FDA's position that periodic overview of SAE data in the public domain is a positive development, and that FDA will work with the GTSAB and will continue to work with OBA and the RAC.
- Dr. Aguilar-Cordova brought up the suggestion by ASGT and others that SAEs be reported in their clinical context. He suggested a possible role for the GTSAB in organizing Gene Transfer Policy



Conferences (GTPCs), and properly disseminating the information put forth at these conferences to the public, investigators, and sponsors.

- Dr. Gordon stated that creation and utilization of the GTSAB and AE database are essential. A usable database in the hands of experts can bring forth important trends in GTR that may prevent an SAE and identify potentially promising areas.
- Dr. Breakefield agreed with Dr. Aguilar-Cordova's comments about the necessity of having mechanisms in place so that knowledgeable people from different sectors of GTR can meet quickly and efficiently, analyze SAE data, and release the analysis publicly. She explained that the existence of more safety nets means a better chance of detecting a potential problem before it becomes serious.
- Dr. Friedmann commented that while the proposal may be imperfect, it does address many of the important issues in the GTR field. He advocated approving the proposal as it is, implementing it, and then being flexible in dealing with problems as they arise. He stressed the importance of the interaction among Government, academia, and industry as being necessary to move the gene transfer field forward.
- Dr. Macklin reminded RAC members that policies rarely include operational details; fine-tuning those details occurs during implementation. She also stated that overlapping responsibilities are not necessarily negative if they result in improved protection of human subjects participating in frontier research areas such as GTR.
- Dr. Greenblatt declared his strong support for the creation of the GTSAB, stating that it would represent a significant improvement over what is currently available and that it has value to patient-subjects and to science. He pointed out that the GTSAB would be reevaluated after two years.

#### **E. Committee Motion 4**

It was moved by Dr. Gordon and seconded by Ms. Levi-Pearl that the RAC recommend the Proposed Action to amend the *NIH Guidelines* to the NIH Director with the understanding that the details will be worked out. The vote was 11 in favor, 0 opposed, and 1 abstention.

#### **VII. Discussion of Human Gene Transfer Protocol #0101-445: Clinical Protocol for Wild-Type p53 Gene Induction in Premalignancies of Squamous Epithelium of the Oral Cavity via an Adenoviral Vector**

Principal Investigator:	Gary Clayman, M.D., University of Texas M.D. Anderson Cancer Center
Sponsor:	Introgen Therapeutics, Inc., represented by Deborah R. Wilson, Ph.D.
RAC Reviewers:	Drs. Aguilar-Cordova, Breakefield, Chow, and Macklin
Ad Hoc Reviewer:	Carter Van Waes, M.D., Ph.D., National Institute on Deafness and Other Communication Disorders, NIH

#### **A. Protocol Summary**

For a discrete group of patients with preneoplastic lesions of the oral cavity, no meaningful treatment exists other than conventional surgery. Surgery does not address the multifocality, high incidence of recurrence, and second primary lesions involving aerodigestive tract sites. Biochemoprevention approaches have demonstrated disappointing results; in more than 50% of patients, lesions become malignant. Biomarker studies have suggested that patients with mutant p53 and genetic instability were at greatest risk of disease progression. The objective of this protocol is to directly modify the precancerous cell to express large quantities of an exogenously introduced, normal tumor suppressor gene product that

may reverse the premalignant process by inducing apoptosis in the cancer predisposed cells, allowing for repopulation with normal genotype epithelial cells. The goal is to determine the transduction efficiency of adenoviral mediated wild-type p53 gene transfer in reversing oral premalignancies.

Patients will receive an injection of the Ad5CMVp53 vector and oral rinse on day 1 followed by twice-daily oral rinses on days 2-5, additional lab work, research blood draws and photo documentation for the completion of one cycle. The study cycle will be repeated on a monthly basis for a period of 6 months. A total of 12 patients will be entered into the phase I dose escalation study with 33 patients anticipated to be entered into the phase II study. Biopsies of normal and preneoplastic tissue are performed at pretreatment and two hours following the first oral rinse of the 1<sup>st</sup> and 6<sup>th</sup> cycles. Alternative biologic endpoints will also be monitored through the collection of serum and urine. Maximal transduction rate will be determined by immunohistochemistry of p53 and downstream gene products.

#### **B. Written Comments From Preliminary Review**

Three RAC members recommended that the protocol warranted public discussion. Drs. Breakefield, Chow, and Macklin submitted written reviews, as did *ad hoc* reviewer Dr. Van Waes, to which the investigators responded in writing and during this meeting.

Dr. Aguilar-Cordova raised no safety concerns. He noted that adenoviruses are relatively unstable at low pH and queried the investigators about the effect of saliva on the adenovirus.

Dr. Breakefield focused on the novel route of administration (oral rinse), which is difficult to model in animals and may have toxic consequences to organs such as the larynx. Because premalignancies were targeted, she was also concerned about the risk-benefit ratio since it was not clear how well the vector would be able to transduce the target cells by this route and, if it did, whether the transduced cells would undergo apoptosis. Given that smoking and alcohol consumption predispose squamous cell carcinomas of the oral cavity, she asked whether participants would be counseled about these risks. Dr. Breakefield also inquired about the stability of the adenoviral vector in saliva, how the saliva will be monitored for shed virus after vector administration, and how SAEs associated with the oral tissues and larynx would be monitored.

Dr. Chow also focused on the route of administration and the targeted disease. She expressed concern about the possible effects of the oral rinse and the 10 percent acetic acid prerinse on nontarget tissues in the oral cavity as well as possible accidental exposure to the epithelial cells lining the airway and the esophagus. Since a control arm using a placebo oral rinse is not proposed, Dr. Chow wondered how investigators would know whether any observed effect was due to the intralesional injection of the virus, the oral rinse, or both.

Dr. Macklin focused on recruitment of participants, how and where it would occur and who would be doing it. She also expressed concerns about the route of administration and the inability to model it in animals prior to human trials. She questioned whether compliance with a 30-minute oral rinsing regimen would be possible, and pointed out that possible harm could result from swallowing or aspirating the virus solution. Overall she expressed concern about the risk-benefit balance, suggesting that the uncertainty of potential benefits may not outweigh the potential harms. In the informed consent document, the terms "patient," "treatment," and "doctor" should be replaced with terms that reflect the experimental nature of the process.

Dr. Van Waes also centered on the use of a new patient population and delivery method. He asked for the percentage of dysplasias that have p53 mutations, the frequency with which lesions with p53 mutations progress to carcinoma, why p53 mutation is not an eligibility requirement, and whether preclinical studies have been performed to support the hypothesis that wt-p53 can efficiently induce apoptosis of premalignant cells and repopulation of normal epithelial cells. He also asked about the rationale and safety of the oral acetic acid rinse, whether acetic acid is a carcinogenic agent in subjects using tobacco and alcohol, and why intralesional injection without the rinse is not being performed first. Dr. Van Waes also suggested that the consent document include a description of the rinse and instructions for research subjects to abstain from oral contact with others.

**C. RAC Discussion**

Ms. Levi-Pearl requested that the informed consent document include financial disclosure information.

Dr. Macklin commented on the "therapeutic misconception" and the need for a clearer distinction in the protocol between the role of researcher and that of a personal physician.

Dr. Van Waes requested that the investigators amend the eligibility criteria to make it clear that they are recruiting participants who have failed other therapies and who have widespread or diffuse disease involvement.

Dr. Friedmann asked the researchers to explain why leukoplakia is not part of the study, and to describe the fate of all the administered adenovirus, particularly whether it survives in the trachea.

**D. Investigator Response**

Dr. Clayman clarified that the protocol is directed toward participants who have failed other standard or experimental treatments. Fifty percent of patients diagnosed with premalignancy progress to the malignancy within 6 months.

In regard to the delivery route, preclinical studies showed no toxicity in mice receiving an equivalent oral dose. Also there have been other trials involving intratumoral injection in which participants have been found to shed the same vector in saliva without ill effect. A 30 minute oral rinse is standard in other treatments for head and neck squamous cell carcinoma patients. The use of the 10 percent acetic acid did not significantly change the pH of the oral cavity, and ingested adenovirus p53 is neutralized by the stomach's pH of 1.

Dr. Clayman explained that leukoplakias are not necessarily premalignant. They can be benign long-term processes that do not progress to malignancy.

**E. Public Comments**

No public comments were offered.

**F. RAC Recommendations**

Dr. Mickelson summarized the following RAC recommendations as follows:

- To revise the eligibility criteria to ensure that only patients with diffuse and refractory premalignancies are enrolled.
- With regard to the informed consent document:
  - To include a financial disclosure for the investigator and any sub-investigators (and if any financial conflict of interests, to give details);
  - To replace the word "patients" with "subjects" or "research participants" since this is clinical research rather than provisional medical care; and
  - To revise the informed consent document to reflect the changes agreed to during the preliminary review (e.g. 30 minute oral rinses would occur in a clinical setting where biohazard containers are available).

**G. Committee Motion 5**

As moved by Dr. Breakefield, the RAC vote on the recommendations was 9 in favor, 0 opposed, and 2 abstentions.

**VIII. Proposed Plan for Addressing Issues Related to Institutional Biosafety Committees/  
Allan Shipp, M.H.A., Office of Biotechnology Activities; Cynthia Dunn, M.D., University of  
Rochester Medical Center; and Andrew George Braun, D.Sc., Harvard Medical School**

The issues for discussion were as follows: (1) Should the *NIH Guidelines* be amended to clarify when an institution conducting recombinant DNA research may use an offsite IBC, defined as an IBC at another institution or a commercial IBC? and (2) Pending such an amendment, should an interim policy be put into place to promote clarity and consistency in the interpretation of the current *NIH Guidelines*?

OBA proposed to hold a conference in fall 2001 on a range of issues pertinent to IBC function. Conference participants will discuss such matters as the origin of IBCs, the meaning and necessity of local review, the importance of community consultation, the role of IBCs relative to IRBs, the relationship of IBCs to Federal agencies, and specific questions directly germane to the offsite IBC question. By opening a dialog on these matters, the conference will inform the development of any necessary amendments to the *NIH Guidelines*.

**A. Mr. Shipp**

Mr. Shipp presented an overview of the membership, procedures, and functions of IBCs as defined in the *NIH Guidelines*. The need to review the current policy has been prompted by two types of queries to OBA. Researchers from institutions that do not have adequate resources to set up their own IBCs would like to use IBCs from neighboring institutions. Investigators who are conducting multisite trials have requested the use of commercial IBCs to coordinate review of the research across sites. A policy interpretation is needed that will optimize subject and community protections and research advancement.

A strawman proposal that included two scenarios was put forth for RAC approval. In scenario A, if an institution or its clinical site conducting GTR were to receive NIH support for recombinant DNA research, it would have to set up a local, institutionally accountable, fully compliant IBC. In scenario B, if an institution or its clinical site did not receive NIH support for recombinant DNA research, but the sponsor of the research did receive NIH support, the site would have to set up its own local, compliant IBC or hire an offsite IBC by contract, with OBA approval. Alternatively, the sponsor IBC could conduct the review, or the sponsor could hire an IBC by contract, with OBA approval. To be acceptable, an offsite IBC used under contract would have to meet the fundamental requirements specified in the *NIH Guidelines* including:

- A majority of the members (three or more) must fulfill the expertise requirements specified in the *NIH Guidelines* (but the expert members need not reside at or be affiliated with the site).
- At least two members must be from the community surrounding the IBC and represent its interests with respect to health and protection of the environment, and these members must be able to consult promptly with other IBC members.
- There must be periodic inspections of the site by the IBC members who have expertise in the type of research being conducted.
- The IBC must be able to be convened as promptly as necessary (which may be done by teleconference).

OBA's ongoing concerns about offsite IBCs included those related to research that occurs in "doc-in-the-box" settings (e.g., in a doctor's office), managing the risks of certain classes of vectors, adequate training of personnel, and ensuring institutional accountability.

**B. Dr. Dunn**

Dr. Dunn described offsite or independent IBCs as only overseeing GTR clinical trials at biosafety levels 1 or 2. Members must have the required expertise but need not be affiliated with the site. Membership will include a biosafety officer, and infection control specialist from the local community to inspect the site. She cited the trend in which clinical research is shifting from academic medical centers to smaller sites

that may not have the professional expertise to support their own IBCs. Independent IBCs could combine the benefits of local review—community awareness and familiarity with the research environment—with that of central coordination—greater access to expertise, and decrease in conflicts of interest because there would be no direct connection to the clinical site. Dr. Dunn urged OBA to issue a clarification statement indicating that compliance with the *NIH Guidelines* regarding IBCs is not dependent on whether the IBC is constituted internally or independently.

**C. Dr. Braun**

Dr. Braun noted that he was speaking for him self, not as a representative of Harvard University.

IBCs were originally established so local communities could become more aware of research in their neighborhoods; therefore, meetings should continue to be open to the public, whether the IBC is internally or externally constituted. At most institutions, serving on an IBC is a difficult job that is rarely rewarded properly. Members are motivated by the interesting work, and knowing that they are working for the good of their institution, their field, and their own consciences. It is unclear whether commercial IBCs could be expected to display the same degree of devotion to their work.

However, some aspects of outside IBCs would be useful: highly specialized knowledge could be made available to small institutions, economies of scale would occur when people work full time on one issue, the potential for conflict of interest among academic colleagues would decrease, and improved cooperation among different sites in the same protocol may occur if a single IBC oversaw the biosafety issues at those multiple sites.

A possible drawback to commercial IBCs would be the creation of a situation in which members have greater loyalty to their employer than to the sponsor, the institution at which the research is being conducted, or the research participants. Also if clinical studies were removed from local IBC responsibility, service on the local IBC would be less interesting, resulting in more difficulty in getting volunteers to serve on the local IBCs.

Dr. Braun summarized his view that outside IBCs can provide a useful role in certain circumstances related to the need to provide expertise in human gene transfer protocols for small clinical establishments. Because there is no substitute for local knowledge or experience, the RAC should strongly encourage clinical sites to establish their own IBCs.

**D. RAC Discussion**

Dr. Friedmann asked for basic information about the Western Institutional Review Board (WIRB). Dr. Dunn responded that the WIRB is an independent company that was established in 1968 to conduct IRB reviews. WIRB members are paid honoraria by either the clinical site or the sponsor on a per-review basis, whether or not the study is approved.

Dr. Juengst pointed out that the definition of "community member" as a local biosafety officer and an infectious disease expert differs from the type of community member added to an academic IBC: a lay person representing the perspective of the surrounding community. Dr. Dunn responded that the community members are familiar with community attitudes, but they are not necessarily lay members. Dr. Mickelson reiterated the concern that the community-member representation should include lay persons from the public.

Dr. Breakfield suggested the possible establishment of regional IBCs to which institutions would contribute expertise. Another important topic for the proposed fall 2001 meeting, for both independent and institutional IBCs, would be a method of public notification of IBC meetings.

Dr. Aguilar-Cordova suggested that the discussion also include how IBCs function for an institutionally affiliated (but geographically distant) research site, especially in light of how community members are involved in the IBC process.

Dr. Patterson asked the RAC for guidance about whether OBA should adopt the proposed strawman

interim policy, adhere to a strict interpretation of the *NIH Guidelines* on this topic, or make decisions on an *ad hoc* basis until the conference. She also asked whether decisions should take into account the level of risk involved.

Dr. Friedmann preferred to postpone major decisions until more information could be learned during the policy conference. However, Dr. Dunn noted that, if the RAC does not make a decision about the use of independent IBCs before the fall of 2001, sponsors seeking to establish IBC review would be prohibited from using investigative sites outside of academic institutions.

Dr. Macklin suggested that the RAC reject a narrow interpretation of IBCs as being "at the clinical site" in favor of IBCs that provide the most expertise. Dr. Breakefield stated that certain protocols would lend themselves more easily—and with more "comfort" within the community—than the use of independent IBCs. Dr. Aguilar-Cordova agreed that not having a strict interpretation of "at the site" for IBCs would be an acceptable interim stance so that OBA could make case-by-case analyses until after the fall 2001 IBC meeting.

Dr. Braun and Dr. Mickelson objected to the statement in the strawman proposal that the meetings of an independent IBC be allowed to be held by teleconference because teleconferencing would defeat the purpose of allowing public participation and involvement.

#### **E. Public Comment**

Dr. J. Tyler Martin, representing Valentis, suggested the need for a "scenario C" to cover sites and sponsors that voluntarily submit to RAC review.

#### **F. Vote of the Committee**

As moved by Ms. Levi-Pearl and seconded by Dr. Aguilar-Cordova, the RAC accepted the outline of the strawman proposal until such time as the proposed IBC conference is held with a friendly amendment regarding teleconferencing. The vote was 7 in favor, 3 opposed, and 0 abstentions.

### **IX. Data Management/Dr. Greenblatt**

Dr. Greenblatt reported that 24 new protocols were submitted to OBA during the December 1 to March 1 reporting period; 22 were exempted from public review by the RAC. Of the 449 total protocols, 38 are classified as gene marking, 409 as gene transfer, and 2 as nontherapeutic in normal volunteers. A breakdown of the 409 GTR protocols indicates that:

- 280 were for cancer.
- 50 were for monogenic diseases (cystic fibrosis was the most frequent).
- 35 were for infectious diseases (all but 1 for HIV).
- 44 were for other diseases (coronary artery disease and peripheral artery disease being the most frequent).

#### **A. Amendments and Updates and Adverse Events**

During the reporting period, 37 amendments and updates were submitted to OBA including annual updates, eligibility criteria updates, and site additions. Three responses to Appendix M-I-C-1 following the initiation of the clinical investigation were also received.

Of the 206 serious or unexpected reports submitted to OBA, 160 were initial reports and 46 were followups; 25 percent of these occurred prior to 2001. Of the 38 reports classified as serious, possibly

associated, and unexpected, 22 were initial reports and 16 were followups.

Dr. Greenblatt described one report in which a research participant received adenoviral p53 gene transfer for ovarian cancer and died a week after receiving the vector. The preliminary autopsy noted severe peritonitis which was possibly related to treatment. However, the final autopsy attributed death to the complications of extensive metastatic carcinoma, changing the AE from possibly related to unrelated.

#### **X. Food and Drug Administration's Proposed Disclosure Rule/Dr. Noguchi**

Dr. Noguchi described FDA's proposed disclosure rule, "Availability for Public Disclosure and Submission to FDA for Public Disclosure of Certain Data and Information Related to Human Gene Therapy or Xenotransplantation," which was published for comment in the January 18, 2001 *Federal Register*. The purpose of the rule is to allow FDA to participate fully in public discussions about GTR and xenotransplantation. While the proposed rule would maintain the confidentiality of information about research participants, trade secrets, and confidential commercial information, it proposes to disclose:

- Product and participant safety data and related information;
- Name and address of the sponsor;
- Clinical indications to be studied;
- A protocol for each planned study, including abstracts, statement of objectives, names and addresses of investigators, names and addresses of official contacts for local review bodies, criteria for subject selection and exclusion, and description of the treatment that will be administered to subjects, as well as the clinical procedures, laboratory tests, or other measures to monitor safety and minimize risk;
- Written informed consent documents;
- Identification of the biological product and method of production;
- Investigational new drug (IND) safety reports;
- Information submitted in the annual report;
- Regulatory status of the IND; and
- Other relevant data and information.

#### **A. RAC Comments**

Dr. Greenblatt asked Dr. Noguchi how this information would be made available to the public. Dr. Noguchi responded that the sponsor will submit redacted information with each official submission to FDA. The redacted information will then be sent to FDA's public dockets, which are publicly available on the Internet and updated daily. Dr. Greenblatt expressed concern that the proposal, if implemented, will make all raw SAE data available, which the RAC has previously stated may not be in the public interest. Considering that this rule would be a major departure from past FDA policy, he asked whether Congress would allow it to take effect. While acknowledging the possibility of Congressional opposition, Dr. Noguchi indicated that the proposed rule is consistent with law enacted in 1902 that ensures public confidence in medical therapies involving biological products.

Dr. Friedmann, Ms. King, and Ms. Levi-Pearl commended FDA for taking this significant step toward greater transparency. Dr. Aguilar-Cordova queried how this proposal would relate to the OBA-proposed database. Dr. Noguchi responded that the proposal is intended to augment the OBA database, and the information released publicly by FDA would be available for inclusion in the OBA database.

Ms. Levi-Pearl urged anyone with an opinion about the proposal to provide public comment during the

comment period. Dr. Noguchi also encouraged comments and noted that the deadline is mid-April 2001.

**B. Public Comments**

**1. Dr. Andrew Braun, Harvard Medical School**

Dr. Braun suggested that the raw data for SAEs need a denominator—the total number of people studied so that the number of SAEs can be put into context. If this background is not provided, reported numbers may be misleading.

**2. Jo Ann Blake, Citizen**

Ms. Blake asked whether SAE data such as that described by Dr. Greenblatt will link directly back to the original document in FDA records. If the proposed rule changes are implemented, Dr. Noguchi responded that this would be possible.

**C. Committee Motion 6**

As moved by Dr. Breakefield and seconded by Ms. King, the RAC voted unanimously (9) to support the implementation of FDA's proposed disclosure rule because it will further the RAC's mandate and is in the public interest.

**XI. Chair's Closing Remarks/Dr. Mickelson**

Dr. Mickelson thanked the RAC members and indicated that the next RAC meeting is scheduled for June 14-15, 2001.

**XII. Adjournment/Dr. Mickelson**

Dr. Mickelson adjourned the meeting at 5:25 p.m. on March 8, 2001.

[Note: Actions approved by the RAC are considered recommendations to the NIH Director; therefore, actions are not considered final until approved by the NIH Director.]

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Amy P. Patterson, M.D.  
Executive Secretary

I hereby acknowledge that, to the best of my knowledge,  
the foregoing Minutes and Attachments are accurate and  
complete.

Date:

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Claudia A. Mickelson, Ph.D.  
Chair



## Attachment I Committee Roster

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## Attachment II

### Attendees

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## Attachment III

### Abbreviations and Acronyms

AAV	adeno-associated virus
AE	adverse event
AIDS	acquired immunodeficiency syndrome
ASGT	American Society of Gene Therapy
BIO	Biotechnology Industry Organization
CC	Clinical Center, NIH
COPR	Council of Public Representatives
DNA	deoxyribonucleic acid
DSMB	Data and Safety Monitoring Board
<i>E. coli</i>	<i>Escherichia coli</i> bacterium
FDA	U.S. Food and Drug Administration
FOIA	Freedom of Information Act
GTPC	Gene Therapy Policy Conference
GTR	gene transfer research
GTSAB	Gene Transfer Safety Assessment Board
HIV-1	human immunodeficiency virus type 1
IBC	Institutional Biosafety Committee
IND	investigational new drug
IRB	Institutional Review Board
LTR	long terminal repeat
MMF	mycophenolate mofetil
NHF	National Hemophilia Foundation
NHLBI	National Heart, Lung and Blood Institute
NIH	National Institutes of Health
<i>NIH Guidelines</i>	<i>NIH Guidelines for Research Involving Recombinant DNA Molecules</i>
OBA	Office of Biotechnology Activities
OD	Office of the Director, NIH
PBMC	peripheral blood mononuclear cells
PBSC	peripheral blood stem cells
PCR	polymerase chain reaction
PI	principal investigator
RAC	Recombinant DNA Advisory Committee
RCR	replication competent retrovirus
RG	risk group
RNA	ribonucleic acid
SAE	serious adverse event
SAER	serious adverse event reporting
TBI	total body irradiation
VSVG	Vesicular Stomatitis Virus G
WIRB	Western Institutional Review Board
wt-HIV	wild-type human immunodeficiency virus

# **EXHIBIT 5**

# **EXHIBIT 6**

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 38/00, 48/00, C12N 15/85</b>	<b>A1</b>	(11) International Publication Number: <b>WO 99/66946</b> (43) International Publication Date: 29 December 1999 (29.12.99)
<p>(21) International Application Number: PCT/US99/14057</p> <p>(22) International Filing Date: 23 June 1999 (23.06.99)</p> <p>(30) Priority Data: 60/090,526 24 June 1998 (24.06.98) US</p> <p>(71) Applicant (for all designated States except US): TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104 (US).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): EL-DEIRY, Wafik, S. [US/US]; Apartment P113, 1500 Locust Street, Philadelphia, PA 19102 (US).</p> <p>(74) Agents: REED, Janet, E. et al.; Dann Dorfman Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103 (US).</p>		<p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: COMPOSITIONS AND METHODS FOR INDUCING APOPTOSIS IN E6-EXPRESSING CELLS</p> <p>(57) Abstract</p> <p>Methods, pharmaceutical compositions and kits are provided for inducing programmed cell death in cells expressing the E6 oncogene. The methods and compositions are particularly suited for treatment of cancers involving infections with E6-expressing virus, such as human papilloma virus (HPV). The methods and compositions utilize the p53 homolog, p73. Unlike p53, p73 is not targeted by the E6 oncoprotein for ubiquitin-mediated degradation, and so provides a viable alternative to p53 therapy for treatment of E6-expressing cancers.</p>		



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**COMPOSITIONS AND METHODS FOR INDUCING  
APOPTOSIS IN E6-EXPRESSING CELLS**

This application claims priority to U.S. Provisional Application 60/090,526, filed June 24, 1998, the entirety of which is incorporated by reference herein.

5

**FIELD OF THE INVENTION**

This invention relates to the field of methods of treatment of cancer. In particular, this invention provides a method of treatment of cancers associated with human papillomavirus infection or other tumors in which the E6 oncogene is expressed, and a pharmaceutical preparation and kit to practice the method.

10

**BACKGROUND OF THE INVENTION**

Various scientific and scholarly articles and patents are referred to in brackets throughout the specification. These articles and patents are incorporated by reference herein to describe the state of the art to which this invention pertains.

15

Infection with human papillomavirus (HPV) is a major risk factor for the development of squamous cell carcinoma of the cervix. The E6-oncoprotein encoded by HPV has been shown to target the tumor suppressor protein p53 for degradation via ubiquitin conjugation and subsequent proteolysis (Scheffner et al., 1990, Cell 63: 1129-1136). HPV-E6-expressing cancer cells are resistant to the tumor suppressive effects of exogenous wild-type

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-2-

p53 delivered by an adenovirus (Ad) vector (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229).

Several approaches have been proposed to control the growth of HPV E6-expressing cancer cells. These include the use of p21-expressing adenovirus to bypass the p53-degradation step (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229), the use of bovine papillomavirus E2 gene to reactivate endogenous p53 (Hwang et al., 1996, Oncogene 12:795-803), the use of hypoxic conditions to suppress p53 degradation (Kim et al., 1997, Cancer Res. 57:4200-4204), the use of alternatively spliced E6 to compete with normally spliced E6 (Pim et al., 1997, Oncogene 15: 257-264), the use of antisense strategies to lower E6 expression (Hamada et al., 1996, Gyn. Onc. 63:219-227; Beer-Romero et al., 1997, Oncogene 14: 595-602), and the generation of p53 mutants resistant to ubiquitin-directed degradation (Crook et al., 1996, Virology, 217:285-292). In the last mentioned approach, it was found that, although lysine mutants of the C-terminus of p53 did resist E6-mediated degradation *in vitro*, the effect was not observed in intact cells, where the lysine mutant was efficiently targeted for degradation (Crook et al., 1996, Virology, 217:285-292). Some tumor-derived mutants of p53 may also be resistant to E6-dependent proteolysis *in vitro* (Medcalf and Milner, 1993, Oncogene 8:2847-2851). p21-expressing adenovirus (Ad-p21) inhibits the growth of E6-over-expressing cells, although the primary effect of p21 over-expression is a growth arrest associated with a large cell phenotype and little, if any, apoptosis (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229; Meng et al., 1998, Clin. Cancer Res. 4: 251-259). Thus, none of the aforementioned approaches has been

-3-

particularly successful in controlling the growth of E6-over-expressing cells by induction of programmed cell death.

Alternative strategies for the suppression of growth of E6-expressing cancer cells are of great utility. Such alternative strategies would ideally induce apoptosis of the E6-over-expressing cells, as well as inhibit cell proliferation.

#### 10 SUMMARY OF THE INVENTION

Therapy based on the p53 tumor suppressor is unavailable for cancers associated with expression of the E6 oncogene because the E6 protein targets p53 for degradation by ubiquitin-mediated proteolysis. It has been discovered in accordance with the present invention that the p53 homolog, p73, is not targeted for degradation by E6 and, moreover, is a potent inhibitor of cancer colony growth and inducer of apoptosis, even in cells that over-express E6. Thus, p73 is a superior tumor suppressor protein for treatment of cancers in which the E6 oncogene is expressed, such as those associated with HPV infection.

According to one aspect of the present invention, method is provided for inducing apoptosis in an E6-expressing cell. The method comprises administering to the cell an amount of p73 protein effective to induce the apoptosis. In one embodiment, the p53 protein is administered as a DNA construct comprising an expressible sequence that encodes the protein. Preferably, the DNA construct is operably inserted into a viral vector for transforming cells.

The method is typically utilized for arresting growth of cancerous cells, particularly cancers

-4-

associated with infection with E6-expressing viruses, such as HPV. In one embodiment, the cell is a cultured cell. In another embodiment, the cell is obtained from the body of a living organism, the administering is performed  
5 ex vivo, and the cell is returned to the living organism. In still another embodiment, the cell is disposed within a living organism and the administering is performed in vivo.

The p73 protein utilized in the method is  
10 preferred to be p73 $\alpha$  or p73 $\beta$ , most preferably the latter. In a preferred embodiment, the protein comprises a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2. If a DNA construct is used, the DNA construct preferably comprises more than 50  
15 nucleotides of SEQ ID NO:3.

According to another aspect of the invention, an apoptotic, E6-expressing transgenic cell is provided, which comprises a heterologous, expressible DNA construct encoding p73. In one embodiment, the cell is obtained  
20 from a cultured cell line. In another embodiment, the cell is a primary cell obtained from a living organism. In yet another embodiment, it is disposed within a living organism.

According to another aspect of the invention, a  
25 pharmaceutical preparation for treatment of cancers associated with E6 over-expression is provided. In one embodiment, the pharmaceutical preparation comprises a p73 protein associated with a delivery vehicle for delivering proteins to cancer cells. In another  
30 embodiment, the preparation comprises an expressible DNA construct encoding p73, associated with a delivery vehicle for delivering DNA to cancer cells. The

-5-

pharmaceutical preparation also may comprise at least one additional active ingredient for treatment of cancer.

According to another aspect of the invention, a kit is provided that contains the pharmaceutical preparation and other optional components. For instance, in a preferred embodiment, the kit may include a second pharmaceutical agent useful for treating cancer.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1.** Ad-E6 infection leads to degradation of both wild-type and mutant p53 in human cancer cells. The human brain (U373, H80; lanes 1-4), breast (SKBr3; lanes 5,6), lung (H460; lanes 7,8), or colon (HCT116, SW480; lanes 9-12) cancer cell lines were infected using Ad-LacZ or Ad-E6 (as indicated). Immunoblotting for p53 expression (upper panels) or pRb expression (lower panels) was carried out as described in Example 1. pRb expression is presented to document equivalent loading between lysates derived from Ad-LacZ and Ad-E6 infected cells. For cell lines that express mutant p53, the following mutations have been previously reported: U373 cell line: R273H (Kaghad et al., 1997, Cell 90: 809-819); SW480 cell line: R273H, P309S (Kaghad et al., 1997, Cell 90: 809-819); SKBr3 cell line: R175H (Kovach et al., 1991, J. Natl. Cancer Inst., 83:1004-1009); H80 cell line (also known as U-373 MG): R273H (Gomez-Manzano et al., 1996, Cancer Res. 56:694-699).

**Figure 2.** p73, unlike p53, is not specifically targeted for degradation in Ad-E6 infected cancer cells. SW480 cells were transfected by p73 $\alpha$  (lanes 1,2), p73 $\alpha$ m

-6-

(lanes 3,4), p73 $\beta$  (lanes 5,6), or p73 $\beta$ m (lanes 7,8). At six hours following transfection, cells were infected by either Ad-LacZ or Ad-E6 (as indicated). At 20 hrs. following infection, expression of p73 $\alpha$  (upper left) or p73 $\beta$  (upper right) was detected by immunoblotting using anti-HA antibody and for p53 (lower panels) expression using anti-p53 antibody, as described in Example 1. The band just above p73 $\alpha$  is a non-specific anti-HA cross-reactive band.

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## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims.

With reference to nucleic acid molecules, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it

30

-7-

exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to proteins or polypeptides, the term "isolated protein (or polypeptide)" or "isolated and purified protein (or polypeptide)" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. For purposes of this invention, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program are the parameters intended to be used herein to compare sequence identity and similarity. Alternatively, standard BLAST query parameters, utilized by public databases such as GenBank, are utilized herein.



-8-

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the protein (i.e. the structure, thermostability characteristics and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of

-9-

the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

Transcriptional and translational control sequences, sometimes referred to herein as "expression control" sequences or elements, or "expression regulating" sequences or elements, are DNA regulatory elements such as promoters, enhancers, ribosome binding sites, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell. The term "expression" is intended to include transcription of DNA and translation of the mRNA transcript.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a

-10-

selectable phenotype such as antibiotic resistance on a transformed cell.

The term "operably linked" means that the regulatory sequences necessary for expression of a particular coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other regulatory elements (e.g., enhancers or translation regulatory sequences) in an expression vector.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, coding sequence is a construct where the coding sequence itself

-11-

is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. For example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

"Killing", "programmed cell death" and "apoptosis" are used interchangeably in this text to describe a series of cellular events that culminates in the death of the target cell. Apoptosis is a characteristic morphological change in which the cell and its nucleus shrink, condense and fragment. Frequently accompanying this morphological change are the activation of intracellular proteases and nucleases that lead to, for example, cell nucleus involution and nuclear DNA fragmentation.

-12-

## II. Description

Human papillomavirus (HPV) is the major cause of cervical cancer worldwide. HPV-E6 protein targets the p53 tumor suppressor protein for degradation by  
5 ubiquitin-mediated proteolysis, making such cancers resistant to p53-mediated therapy.

In accordance with the present invention, two discoveries have been made that have significant implications and suggest novel strategies for cancer  
10 therapy. The first discovery is that HPV-E6 targets both endogenous wild-type and mutant p53 for degradation (Fig. 1). Possibly because p53 mutations are rare in cervical cancer (Busby-Earle et al., 1994, Br. J. Cancer 69: 732-737) the hypothesis that HPV-E6 could target  
15 endogenous mutant p53 for degradation has not been previously directly tested. While several studies have reported low levels of p53 expression and an inverse correlation between the presence of HPV and p53 expression (Scheffner et al., 1991, Proc. Natl. Acad.  
20 Sci. USA 88: 5523-5527; Srivastava et al., 1992, Carcinogenesis 13: 1273-1275; Baret et al., 1996, Eur. J. Gyn. Onc. 17: 283-285; Hachisuga et al., 1996, Pathology 28: 28-31), there is apparently no such correlation between p53 mutation and HPV (Busby-Earle et al., 1994,  
25 Br. J. Cancer 69: 732-737; Kim and Kim, 1995, Yonsei Med. J. 36:412-425). While the discovery that HPV-E6 also targets mutant p53 for degradation provides no insight into how the rare p53 mutations may contribute to HPV-associated cervical cancer, it is consistent with the  
30 known inverse correlation between p53 expression and the presence of HPV in high risk cervical cancer.

The second and more significant discovery is that the p53 homolog, p73, is not targeted for

-13-

degradation by the E6 oncoprotein. Furthermore, as described in greater detail below and in Example 1, p73 is a potent inducer of apoptosis and is an effective inhibitor of cancer cell growth. For such HPV

5 E6-expressing cancers where p53 is degraded and fails to control growth, p73 is an excellent substitute for p53 in gene replacement because of its resistance to E6-mediated proteolysis. Other differences of effect of viral oncoproteins have been noted (Marin et al., 1998, Mol. Cell. Biol. 18:6316-6324; Steengenga et al., 1999, Mol. Cell. Biol. 19:3885-3894; Dobbelstein and Roth, 1998, J. Gen Virol. 79:3079-3083; Roth et al., 1998, J. Virol. 72:8510-8516; Reichelt et al., 1999, Arch. Virol. 144:621-626). It is noteworthy that, even though p73 has  
10 the potential to interact with p53 in a yeast two-hybrid analysis (Kaghad et al., 1997, Cell 90: 809-819), the expressed p73 is not subject to E6-dependent proteolysis under conditions where high levels of endogenous mutant p53 are degraded (Fig. 2).

20 Provided with this invention are methods, pharmaceutical preparations and kits that utilize p73 for arresting the growth of E6-expressing cells, particularly HPV-infected cancer cells. The treatment of the target cells may be *in vivo*, within the patient; or *ex vivo*,  
25 removed from the patient, treated, and reintroduced into the patient. It is contemplated that the methods, pharmaceutical preparations and kits of the invention can be used alone or in conjunction with chemotherapy or radiation therapy to treat cancers *in vivo*.  
30 Additionally, the methods, pharmaceutical preparations and kit of the invention can be used for experimental purposes *in vitro* with standard cell cultures.

-14-

As mentioned, the treatment of cancers associated with the over-expression of E6 protein is of particular interest. Several circumstances may result in mammalian cells that over-express E6 protein. Commonly, this nature of cell results from an infection with human papillomavirus (HPV) wherein the E6-oncogene encoded by the virus is expressed in the cell. HPV infection is well-known to result in cancers of the uterine cervix. In addition to anogential cancer, HPV infection may also result in esophageal squamous cell cancer, laryngeal papilloma, bronchiolo-alveolar carcinoma, penile carcinoma and bladder carcinoma, among others. Additionally, E6-over-expression may also result from a mutation in the mammalian cell genome such that the endogenous E6 gene is over-expressed. All mammalian cells that over-express the E6 protein, regardless of the origin of the phenotype, are contemplated for treatment with the method of the invention.

The following description set forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1999) (hereinafter "Ausubel et al.") are used.

Any p73 variant and the nucleic acid sequence encoding it are considered suitable for use in the present invention. In this regard, it should be noted that the two major splice variants of p73, p73 $\alpha$  and p73 $\beta$ ,

-15-

both have been found resistant to E6-mediated degradation (see Example 1), though p73 $\beta$  appears to be somewhat more effective in this regard and is preferred for the practice of the present invention.

5           The amino acid sequence of p73 protein on which to base the nucleic acid construct is ideally from the gene that is endogenous to the species which is being treated. In a preferred embodiment, *Homo sapiens* is being treated and the nucleic acid construct encodes SEQ  
10 ID NO:1 or SEQ ID NO:2. In a most preferred embodiment, the nucleic acid sequence is SEQ ID NO:3. Other variants of p73 protein also exist in *Homo sapiens* and the sequences of these variants are also contemplated for use with the invention (DeLaurenzi et al., 1999, Cell Death  
15 Differ. 6:389-390 incorporated by reference herein; Genbank Accession No. Y11416 incorporated by reference herein).

          The availability of amino acid sequence information, such as the full length sequence in SEQ ID  
20 NO:1 and SEQ ID NO:2 enables the preparation of a synthetic gene that can be used to synthesize the *Homo sapiens* p73 protein in standard *in vivo* expression systems or to make viral vectors expressing the p73 protein. The sequence encoding *Homo sapiens* p73 from  
25 isolated native nucleic acid molecules such as SEQ ID NO:3 can be utilized. The amino acid and nucleic acid sequences found in Genbank Accession Nos. AF138873, Y11419 and AF043641 can be used to prepare the p73 protein endogenous to *Mus musculus*, *Chlorocebus aethiops*  
30 and *Barbus barbus*, respectively. Alternately, an isolated nucleic acid that encodes the amino acid sequence of the invention can be prepared by oligonucleotide synthesis. Codon usage tables can be



-16-

used to design a synthetic sequence that encodes the protein of the invention. In a preferred embodiment, the codon usage table has been derived from the organism in which the synthetic nucleic acid will be expressed. For example, the codon usage for *E. coli* would be used to design an expression DNA construct to produce the *Homo sapiens* p73 in *E. coli*.

Synthetic oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant oligonucleotide may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC).

Nucleic acid molecules encoding p73 also may be isolated from appropriate species using methods well known in the art. Native nucleic acid sequences may be isolated by screening mammalian or other cDNA or genomic libraries with oligonucleotides preferably designed to match the *Homo sapiens* coding sequence of p73 (SEQ ID NO:3). Several other p73 amino acid sequences are now known: *Mus musculus*, Genbank Accession No. AF138873; *Chlorocebus aethiops* (Green Monkey), Genbank Accession No. Y11419; and *Barbus barbus*, Genbank Accession No. AF043641; each of these sequences is incorporated by reference herein. Oligonucleotides designed to match any of these sequences or to match regions of high homology between these sequences may also be used to screen for mammalian p73-encoding nucleotides. In positions of degeneracy where more than one nucleic acid residue could be used to encode the appropriate amino acid residue, all the appropriate nucleic acids residues may be incorporated to create a mixed oligonucleotide population, or a neutral base such as inosine may be

-17-

used. The strategy of oligonucleotide design is well known in the art (see also Sambrook et al., Molecular Cloning, 1989, Cold Spring Harbor Press, Cold Spring Harbor NY). Alternatively, PCR (polymerase chain

- 5 reaction) primers may be designed by the above method to match a known coding sequence of p73, and these primers used to amplify the native nucleic acids from isolated mammalian cDNA or genomic DNA.

- Nucleic acids having the appropriate sequence  
10 homology with a *Homo sapiens* p73 synthetic nucleic acid molecule may be identified by using hybridization and washing conditions of appropriate stringency. One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid  
15 molecules of a specified sequence homology (Sambrook et al., 1989, *supra*):

$$T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{G+C}) - 0.63 (\% \text{formamide}) - 600/\text{\#bp in duplex}$$

- 20 As an illustration of the above formula, using  $[\text{Na}^+] = [0.368]$  and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the  $T_m$  is 57°C. The  $T_m$  of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than  
25 about 75% sequence identity would be observed using a hybridization temperature of 42°C.

- Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid  
30 cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell.

-18-

P73 protein can be produced by using *in vitro* expression methods known in the art. For example, part or all of a DNA molecule, such as a DNA encoding the amino acid sequence SEQ ID NO:1 or SEQ ID NO:2, may be  
5 inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or a eukaryotic cell, such as *Saccharomyces cerevisiae* or other yeast. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the  
10 recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by  
15 immunological interaction with antibodies that bind specifically to the recombinant protein or fusion proteins such as His tags. Such methods are commonly used by skilled practitioners.

The method of the invention for treating  
20 mammalian cells that over-express E6 comprises administering a therapeutically effective amount of p73 protein to the target cells. The administration of the p73 protein can be accomplished via several methods, including the exposing the target cell, i.e., the E6-  
25 over-expressing cell, to p73 protein, or exposing the target cell to a nucleic acid construct that expresses an appropriate p73 coding sequence.

Any method of administration of p73 (e.g, as a protein or as a nucleic acid encoding the protein) is  
30 appropriate as long as it results in increased levels of p73 protein within the target cell. The choice of method of administration will depend largely on the position of the target cells and the length of time the treatment is

-19-

needed. Target cells may be removed from the patient and treated *ex vivo*, and then reintroduced to the patient. Additionally, the treatment may be used in cell cultures for experimental purposes. In a preferred embodiment, the target cells comprise E6-over-expressing carcinomas. In a more preferred embodiment, the target cells are papilloma-virus positive cancers. In a most preferred embodiment, the target cells are HPV-positive carcinomas of the uterine cervix.

10           The administration of p73 protein to target cells can be accomplished by exposing the target cell to p73 protein. When the target cell are tumor cells within an animal, it is preferred that the protein is administered in a protected form to increase their stability cells One strategy of accomplishing this is to use liposomes. Liposomes are water-filled vesicles composed of several phospholipids layers surrounding an aqueous core with an outer shell capable of providing direction to specific target cells. Typically liposomes are composed of some combination of phosphatidylcholine, cholesterol, phosphatidylglycerol or other glycolipids or phospholipids (Hudson and Black, 1993, American Pharmacy NS33(5):23-24). Insoluble polymers composed of polyethylene may also be used to form a protective layer around the protein, inhibiting degradation while traveling to the target cell (Hudson and Black, 1993, American Pharmacy NS33(5):23-24). Another way to deliver p73 protein to target cells is to couple the protein to a target cell-specific monoclonal antibody. This approach allows the protein to be specifically delivered to the target cell and minimizes toxic effects on non-target cells (Houston, 1993, Current Opinion in Biotechnology 4:739-744).

-20-

In preferred embodiments, the p73 protein is administered to the target cell through the use of heterologous nucleic acids that will cause the protein to be synthesized within the target cell. These nucleic acids can be temporary residents in the target cell, such as expression plasmids, or they can be stably integrated into the genome of the target cell. Expression plasmids are particularly appropriate for experimental work with cell cultures, such as illustrated in Example 1. The construction of such plasmids and the transformation of target cells with them *in vitro* is well known to those of skill in the art of cell biology. Expression vectors suitable for p73 expression in mammalian cells are commercially available (Gene Therapy Systems, San Diego). Naked DNA and plasmids may be delivered to the target cells by several known means. The naked DNA may be transferred directly into the genetic material of the cells (Wolff et al., 1990, Science 247:1465-1468), the p73-encoding DNA may be delivered in liposomes (Ledley, 1987, J. Pediatrics 110:1) or proteoliposomes that contain viral envelope receptor proteins (Nicolau et al, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:1068), or the p73-encoding DNA may be coupled to a polylysine-glycoprotein carrier complex.

For a longer lasting expression of p73 within target cells, viral vectors are preferred. A variety of viral vector may be used in this invention, included retroviral vectors such as the herpes simplex virus (U.S. Patent 5,288,641, incorporated herein by reference), Cytomegalovirus, murine leukemia virus (Blaese et al., 1995, Science 270:475-479) and similar as described by Miller (Miller, 1992, Curr. Top. Microbiol. Immunol. 158:1). Recombinant adeno-associated virus (AAV vectors)

-21-

such as those described by U.S. Patent No. 5,139,941 (which is incorporated herein by reference) and recombinant adenoviral vectors (He et al., 1998, PNAS 95:2509-2514, incorporated by reference herein) are particularly preferred. Also contemplated are recombinant lentivirus vectors such as a recombinant Human Immunodeficiency Virus (U.S. Patent No. 5,885,805; Blaese et al., 1995, Science 270:475-479; Onodera et al., 1998, J. of Virology 72:1769-1774) and Feline Immunodeficiency Virus. Often these vectors have been designed so that they are replication-defective, and the techniques to prepare such vectors are well known in the art (Ghosh-Choudhury and Graham, 1987, Biochem. Biophys. Res. Comm. 147:964-973; McGrory, W. J. et al., 1988, Virology 163:614-617; Gluzman et al., 1982 in Eukaryotic Viral Vectors (Gluzman, Y., Ed.) pp. 187-192, Cold Spring Harbor Press, Cold Spring Harbor, N.Y). It is also contemplated that viral vectors that are replication competent may be used to improve the efficacy of the treatment of solid tumors (Wildner et al., 1999, Gene Ther. 6:57-62).

The recombinant vector of the invention comprises a nucleic acid construct comprising a sequence encoding a p73 protein operably linked to an appropriate promoter and other expression-regulatory sequences. For treatment of cancer cells, a strong constitutive promoter, such as a cytomegalovirus promoter, a viral LTR, RSV or SV40 promoter, is preferred. In a preferred embodiment, a cytomegalovirus promoter is used. Additionally, promoters associated with genes that are expressed at high levels in mammalian cells, such as elongation factor-1 and actin, are also contemplated. It is particularly advantageous to use a viral-specific and

-22-

-regulated promoter to direct expression specifically in affected cancer cells. In a particularly preferred embodiment, the HPV-E6 promoter is used.

In a particularly preferred embodiment, a  
5 recombinant adenoviral vector is used to deliver the p73-expressing construct to the target cell. The use of adenoviral vectors for gene therapy is well known in the art (El-Deiry et al., 1993, Cell 75:817; Blogosklonny and El-Deiry, 1996, Int. J. Cancer 67:386-395; Prabhu et al.,  
10 1996, Clin Cancer Res. 2:1221-1230; Zeng et al., 1997, Int. J. Oncol. 11:221-226; Mitchell and El-Deiry, 1999, Cell Growth and Diff. 10:223-230; Meng et al., 1998, Clin. Cancer Res. 4:251-259; Blagosklonny and El-Deiry, 1998, Int. J. Cancer 75:933-940). In particular, an  
15 adenovirus vector has been used successfully to deliver p53 to target cells to treat lung cancer in human patients (Roth et al., 1996, Nature Med. 2:974 incorporated herein by reference; and U.S. Patent 5,747,469 incorporated herein by reference). It is  
20 contemplated that these protocols with simple variation that will be well known to those in the art can be used to administer the p73 protein to target cells in the invention. In a most preferred embodiment, therapeutically effective amounts of the viral vector are  
25 delivered to the cancers by direct injection.

The interchangeability of p53 and p73 in these methods arises from the high degree of similarity that these proteins have, both in structure and function. p53 and p73 have significant amino acid sequence similarities  
30 (Kaghad et al, 1997, Cell 90:809-818, incorporated by reference herein), particularly in the most conserved regions of p53: the transactivation, DNA binding and p53 oligomerization domains. A sequence similar to the MDM2-

-23-

binding domain is also present in p73. The residues in p53 often found mutated in tumors and shown to be required for DNA recognition are conserved and occupy identical positions in p73. The C-terminal domain of p73 $\alpha$  shows homology to invertebrate p53 homologs. Finally the intron-exon organization of p73 is very similar to p53.

p53 and p73 are also functionally similar. Both display homotypic interactions, and p53 and p73 $\beta$  display significant mutual interactions (Kaghad et al, 1997, Cell 90:809-818). Both are inhibited by adenovirus E4ORF6 (Higashino et al., 1998, PNAS 95:15683-15687) and the MDM2 oncoprotein (Zeng et al., 1999, Mol. Cell. Biol. 19:327-3266; Dobbelstein et al., 1999, Oncogene 18:2101-2106). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells in a manner similar to p53 (Di Como et al., 1999, Mol. Cell. Biol. 19:1438-1449). p73 regulates p53 target genes when p73 is over-expressed in cells (Zhu et al., 1998, Cancer Research 58:5061-5065; Jost et al., 1997, Nature 389:181-184). Finally, as a result of activation of p53-responsive genes, p73 can inhibit cell growth and induce apoptosis in a manner similar to p53.

Also provided with the invention are pharmaceutical compositions that can be used to treat mammalian cells with p73 *in vitro*, *in vivo* and *ex vivo*. The compositions comprise either p73 protein or nucleic acids encoding p73 protein. The pharmaceutical compositions of the invention are formulated in an appropriate "biologically acceptable medium". As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media and the like which may be appropriate for the desired route of administration of



-24-

the pharmaceutical preparation, as exemplified in the preceding paragraph. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is  
5 incompatible with the nucleic acid molecules or proteins to be administered, its use in the pharmaceutical preparation is contemplated.

The pharmaceutical preparation is formulated in dosage unit form for ease of administration and  
10 uniformity of dosage. Dosage unit form, as used herein, refers to a physically discrete unit of the pharmaceutical preparation appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to produce the  
15 desired effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate dosage unit are well known to those skilled in the art.

The pharmaceutical composition also can include  
20 various other components as additives or adjuncts. Exemplary pharmaceutically acceptable components or adjuncts which are employed in relevant circumstances include antioxidants, free radical scavenging agents, peptides, growth factors, antibiotics, bacteriostatic  
25 agents, immunosuppressives, anticoagulants, buffering agents, anti-inflammatory agents, anti-pyretics, time release binders, anaesthetics, steroids and corticosteroids. Such components can provide additional therapeutic benefit, act to effect the therapeutic action  
30 of the pharmaceutical composition, or act towards preventing any potential side effects which may be posed as a result of administration of the pharmaceutical composition. In certain circumstances, the p73 protein

-25-

or nucleic acid molecule can be employed as part of a pharmaceutical composition with other compounds (e.g., chemotherapeutic agents) intended to prevent or treat cancer or a related disorder.

5           The manner in which the pharmaceutical preparations are administered can vary. They can be administered by inhalation (e.g., in the form of an aerosol either nasally or using delivery articles of the type set forth in U.S. Patent No. 4,922,901 to Brooks et  
10 al.); topically (e.g., in lotion form or as a suppository); orally (e.g., in liquid form within a solvent such as an aqueous or non-aqueous liquid, or within a solid carrier); intravenously (e.g., within a dextrose or saline solution); as an infusion or injection  
15 (e.g., as a suspension or as an emulsion in a pharmaceutically acceptable liquid or mixture of liquids); intrathecally; intracerebro- ventricularly; or transdermally (e.g., using a transdermal patch). Exemplary methods for administering such compounds will  
20 be apparent to the skilled artisan. The administration of the pharmaceutical compositions of the present invention can be intermittent, or at a gradual, continuous, constant or controlled rate to a warm-blooded animal, (e.g., a mammal such as a mouse, rat, cat,  
25 rabbit, dog, pig, cow, or monkey); but advantageously is preferably administered to a human being. In addition, the time interval between administrations can vary. Administration preferably is such that the active ingredients of the pharmaceutical formulation contact the  
30 target cells, whether within or outside the body of a mammalian subject.

The appropriate dose of the compound is that amount effective to result in increased levels of p73

-26-

protein within the target cell. By "effective amount", "therapeutic amount" or "effective dose" is meant that amount sufficient to elicit the desired pharmacological or therapeutic effects, thus resulting in effective  
5 prevention or treatment of the disorder. Prevention of the disorder is manifested by delaying the onset of the symptoms of the disorder. Treatment of the disorder is manifested by a decrease in the symptoms associated with the disorder or an amelioration of the recurrence of the  
10 symptoms of the disorder.

The effective dose can vary, depending upon factors such as the condition of the patient, the severity of the symptoms of the disorder, and the manner in which the pharmaceutical composition is administered.  
15 The effective dose of compounds will of course differ from patient to patient but in general includes amounts starting where target cell growth is halted to where the target cell is killed. Dosages contemplated for use with the retroviral vector embodiment of the invention are  
20 those suggested in U.S. Patent 5,747,469 (incorporated herein by reference). One of ordinary skill in the art will know how to determine such doses without undue experimentation.

Kits with the components necessary to treat E6-  
25 over-expressing target cells by the method of the invention are also provided. In a preferred embodiment, the kit contains therapeutically effective amounts of the pharmaceutical preparation of the invention in a container. The pharmaceutical preparation in the kit may  
30 be comprised of p73 protein or a DNA construct encoding p73, preferably inserted into a vector for transforming cells. The p73 protein or p73 encoding viral vector may be in the form of a pharmaceutically acceptable sterile

-27-

solution such as sterile saline, dextrose solution or buffered solution. Alternatively, the p73 protein or p73 encoding viral vector can be lyophilized or desiccated. In this instance the kit may optionally further comprise  
5 a container of a pharmaceutically acceptable solution, (e.g., saline, dextrose solution, etc.), preferably sterile, to reconstitute the pharmaceutical preparation to form a solution for injection purposes. Optionally, instructions may be included in the kit. The kit may  
10 additionally comprise pharmaceutical preparations in containers for other therapies related to cancer treatment, such as chemotherapy.

The following example is provided to describe  
15 the invention in greater detail. It is intended to illustrate, not to limit, the invention.

#### EXAMPLE 1

20 p73 $\alpha$  and p73 $\beta$  Suppress Growth and Induce Apoptosis in Human Papilloma Virus E6-Expressing Cancer Cells

##### Materials and Methods

**Plasmids.** The mammalian expression vector pCMV-neo-Bam (Baker et al., 1990, Science 249: 912-915)  
25 and the wild-type p53 expression vector SN3 (Baker et al., 1990, Science 249: 912-915) were obtained from Bert Vogelstein (Johns Hopkins University). Wild-type and mutant p73 $\alpha$  and p73 $\beta$  plasmids (Jost et al., 1997, Nature 389: 191-194; incorporated herein by reference) were  
30 obtained from William G. Kaelin, Jr. (Dana Farber Cancer Institute). The HPV-E6 expression plasmid (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229; incorporated herein by reference) was obtained from Kathleen Cho (Johns Hopkins University).

-28-

**Cell culture and transfection conditions.** The mutant p53-expressing human colon adenocarcinoma cell line SW480 was maintained in culture as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). The mutant p53-expressing human glioma cell lines U373 and H80 were obtained from Peter C. Phillips (The Children's Hospital of Philadelphia) and the wild-type p53-expressing human non-small cell lung cancer cell line H460 was obtained from Stephen B. Baylin (Johns Hopkins University). Mutant p53-expressing SKBr3 cells were obtained from American Type Culture Collection (Rockville, MD). SW480 cells were transfected using Lipofectin (BRL) as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). At 20 hrs. following transfection, cells were harvested and protein lysates electrophoresed through 10% polyacrylamide gels and immunoblotted as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). Analysis of p53 expression was performed using the anti-human p53 monoclonal antibody pAb1801 (Ab2; Oncogene Science). For detection of exogenous p73 protein expression, the anti-HA antibody was used as previously described (Jost et al., 1997, Nature 389: 191-194).

**Adenovirus infections.** The Ad-LacZ reagent (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229) was obtained from Bert Vogelstein. The HPV type 16 E6-expressing replication deficient adenovirus was prepared and titered as previously described (Satyamoorthy et al., 1997, Cancer Res. 57: 1873-1876; Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). Briefly, the CMV promoter-driven HPV type 16 E6 cDNA was inserted into an E3-deleted adenovirus by homologous recombination to generate E1 and E3 deleted replication

-29-

defective Ad-E6 adenovirus (Satyamoorthy et al., 1997, Cancer Res. 57: 1873-1876; incorporated herein by reference). The cloned HPV-E6 DNA sequence was verified and expression of HPV-E6 was verified by Northern  
5 blotting of total RNA derived from Ad-E6 versus Ad-LacZ infected cells. Cells were infected using an MOI of 50 as previously described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). Infection of SW480 cells using Ad-LacZ at an MOI of 50 followed by X-gal staining  
10 revealed greater than 99% infectivity.

**Colony suppression assays.** Transfections were carried out as described above except that the tumor suppressive (p53 or p73) or control (pCMV-neo-Bam) plasmid represented 80% of the total DNA and the  
15 degrading (pCMV-E6) or control (pCMV-neo-Bam) represented the remaining 20% of the total DNA. At 24 hrs following transfection, G418 selection was begun using 1 mg/ml as a final concentration. Selection was continued for 7-12 days and colony growth was analyzed as previously  
20 described (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229).

**TUNEL assays.** At 48 hrs following transfection, cells were formalin fixed and the extent of apoptosis was assessed by nicked-end labeling using the  
25 Apotag kit (Oncor) followed by analysis using fluorescence microscopy.

## Results

**HPV-E6 targets both wild-type and mutant p53 protein for degradation.** Using an E6-expressing  
30 adenovirus (Ad-E6) a panel of human cancer cells derived from different tissues and containing either endogenous wild-type or mutant p53 were infected (Fig. 1). As

-30-

compared with Ad-LacZ infected cells, E6-expressing cells expressed substantially reduced levels of either wild-type or mutant p53 protein (Fig. 1, compare even to odd lanes). Thus the HPV-E6 protein targets both wild-type and mutant p53 for degradation. E6 does not target the cell cycle regulatory proteins pRb, p21, cyclin E or p27 for degradation (Fig. 1 lower panels). The phosphorylation state of Rb in Ad-E6 was not altered as compared to Ad-LacZ infected cells (Fig. 1 lower panels), regardless of the p53 status of the cells.

**p73 is resistant to HPV E6-dependent proteolysis.** Because p53 is degraded in HPV-E6 expressing cancer cells, such cells are not ideally suited for gene replacement therapy (Prabhu et al., 1996, Clin. Cancer Res. 2: 1221-1229). In HPV E6-expressing cancer cells, p53 is degraded while exogenous p73 is resistant to E6-targeting to the proteasome (Fig. 2). The resistance of p73 to E6-dependent proteolysis was observed with p73 $\alpha$ , p73 $\alpha$ m, p73 $\beta$ , or p73 $\beta$ m. This observation suggested that p73 is a candidate for gene replacement in E6-expressing cancer cells.

**p73 $\beta$  induces apoptosis and suppresses growth in HPV E6-expressing human cancer cells.** p73 $\beta$  was previously found to be a potent activator of p53-dependent gene expression (Kaghad et al., 1997, Cell 90: 809-819; Jost et al., 1997, Nature 389: 191-194). p73 $\beta$  in colony suppression assays in the absence or presence of E6-expression. Whereas p53 failed to inhibit the growth of E6-expressing cancer cells, p73 $\beta$  was found to be a potent growth suppressor. Transfection studies revealed that p73 $\alpha$  was a less potent suppressor of growth of SW480 cancer cells either in the absence or presence of HPV-E6.

-31-

This could not be explained by dominant negative inhibition of p73 $\alpha$  by the endogenous p53 mutant in SW480 cells because it was previously shown that p73 $\alpha$  shows negligible interaction with p53 (Kaghad et al., 1997, 5 Cell 90: 809-819).

p73 $\beta$  has been previously shown to be an inducer of apoptosis, similar to p53 (Jost et al., 1997, Nature 389: 191-194). Whereas p53-dependent apoptosis was inhibited in E6-expressing cells, p73 $\beta$  was still capable 10 of inducing apoptosis similar to what is observed in the absence of E6. Therefore the colony suppression phenotype observed following p53 or p73 $\beta$  expression in the presence or absence of E6 can be explained by their ability to induce apoptosis under these conditions.

15 These results suggest that the p73 $\beta$ -dependent suppression of growth of HPV E6-expressing cancer cells occurs through an apoptotic mechanism.

The present invention is not limited to the 20 embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.



What is claimed:

1. A method of inducing apoptosis in an E6-expressing cell, comprising administering to the cell an amount of p73 protein effective to induce the apoptosis.

2. The method of claim 1 wherein the p53 protein is administered as a DNA construct comprising an expressible sequence that encodes the p73 protein.

10

3. The method of claim 2, wherein the DNA construct is operably inserted into a viral vector.

4. The method of claim 3, wherein the viral vector is selected from the group consisting of adenoviral vectors, HIV vectors, FIV vectors, herpes viral vectors, adeno-associated vectors and cytomegaloviral vectors.

5. The method of claim 1, wherein the cell is a cancerous cell.

6. The method of claim 1, wherein the cell is infected with Human papilloma virus.

25

7. The method of claim 1, wherein the cell is a cultured cell.

8. The method of claim 1, wherein the cell is obtained from the body of a living organism, the administering is performed ex vivo, and the cell is returned to the living organism.

30

9. The method of claim 1, wherein the cell is disposed within a living organism and the administering is performed *in vivo*.

5           10. The method of claim 1, wherein the cell is obtained from a species selected from the group consisting of *Homo sapiens*, *Mus musculus* and *Chlorocebus aethiops*.

10           11. The method of claim 1, wherein the p73 protein is p73 $\alpha$  or p73 $\beta$ .

12. The method of claim 11, wherein the protein comprises a sequence selected from the group  
15 consisting of SEQ ID NO:1 and SEQ ID NO:2.

13. The method of claim 2, wherein the DNA construct comprises more than 50 nucleotides of SEQ ID  
20 NO:3.

14. A pharmaceutical preparation for treatment of cancers associated with E6 over-expression, comprising p73 protein associated with a delivery vehicle for delivering the preparation to cancer cells.

25           15. The pharmaceutical preparation of claim 14, wherein the p73 protein is p73 $\alpha$  or p73 $\beta$ .

16. The pharmaceutical preparation of claim  
30 15, wherein the protein comprises a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

17. The pharmaceutical preparation of claim 14, which further comprises at least one additional active ingredient for treatment of cancer.

5           18. A kit comprising a container containing one or more dosage units of the pharmaceutical composition of claim 14.

10           19. The kit of claim 18, which further comprises at least one additional pharmaceutical agent for treatment of cancer.

15           20. A pharmaceutical preparation for treatment of cancers associated with E6 over-expression, comprising an expressible DNA construct encoding p73, associated with a delivery vehicle for delivering the preparation to cancer cells.

20           21. The pharmaceutical preparation of claim 20, wherein the DNA construct is operably inserted into a vector for transforming cells.

25           22. The pharmaceutical preparation of claim 21, wherein the vector is a viral vector selected from the group consisting of adenoviral vectors, HIV vectors, FIV vectors, herpes viral vectors, adeno-associated vectors and cytomegaloviral vectors.

30           23. A kit comprising a container containing one or more dosage units of the pharmaceutical composition of claim 20.

24. The kit of claim 23, which further comprises at least one additional pharmaceutical agent for treatment of cancer.

5           25. An apoptotic, E6-expressing transgenic cell comprising a heterologous, expressible DNA construct encoding p73.

26. The cell of claim 25, obtained from a  
10   cultured cell line.

27. The cell of claim 25, disposed within a living organism.

15           28. The cell of claim 25, from a species selected from the group consisting of *Homo sapiens*, *Mus musculus* and *Chlorocebus aethiops*.

1/2



Figure 1

2/2

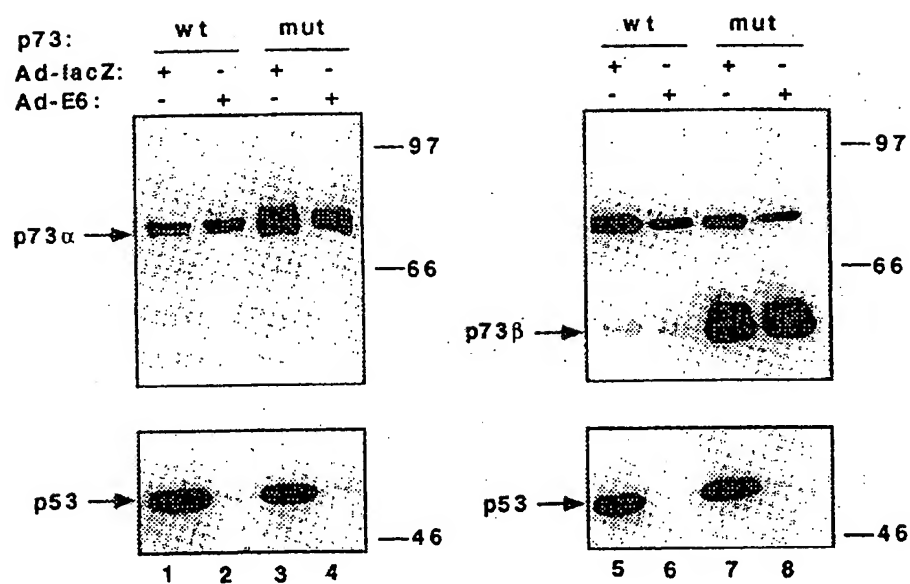


Figure 2

## SEQUENCE LISTING

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/14057

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 38/00, 48/00; C12N 15/85

US CL : 514/2, 44; 424/93.21, 93.3; 435/455, 456

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 44; 424/93.21, 93.3; 435/455; 456

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS: MEDLINE, BIOSIS, EMBASE, CAPLUS, BIOTECHDS

search terms: p73, c6, papilloma, aethiops

SEQUENCE SEARCH; SEQ ID NOS 1-3

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	Database Medline, AN 1998290825, PRABHU et al. p73beta, unlike p53, suppresses growth and induces apoptosis of human papillomavirus E6-expressing cancer cells. International Journal of Oncology. July 1998. Vol. 13. pages 5-9, abstract only.	1-28
A	JOST et al. p73 is a human p53-related protein that can induce apoptosis. Nature. 11 September 1997. Vol. 389. pages 191-194, entire document.	1-28
A	KAGHAD et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell. August 1997. Vol 90. pages 809-819, entire document.	1-28

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

07 SEPTEMBER 1999

Date of mailing of the international search report

28 OCT 1999

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/14057

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	MARIN et al. Viral oncoproteins discriminate between p53 and the p53 homologue p73. Molecular and Cellular Biology. November 1998. Vol. 16, pages 6316-6324, entire document.	1-28
A	OREN. Lonely no more: p53 finds its kin in a tumor suppressor haven. Cell. September 1997. Vol 90. Pages 829-832, entire document.	1-28

# **EXHIBIT 7**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>A61K 47/10, 47/42, 48/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/29024</b> <b>(43) International Publication Date:</b> 25 May 2000 (25.05.00)
<b>(21) International Application Number:</b> PCT/US99/27177 <b>(22) International Filing Date:</b> 16 November 1999 (16.11.99)  <b>(30) Priority Data:</b> 60/108,606                      16 November 1998 (16.11.98)      US 60/133,116                      7 May 1999 (07.05.99)                      US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications</b> US    60/108,606 (CON) Filed on                                      16 November 1998 (16.11.98) US    60/133,116 (CON) Filed on                                      7 May 1999 (07.05.99)  <b>(71) Applicant (for all designated States except US):</b> INTROGEN THERAPEUTICS, INC. [US/US]; Suite 1850, 301 Congress Avenue, Austin, TX 78701 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ZHANG, Shuyuan [CN/US]; D-106, 274 Glen Riddle Road, Media, PA 19063 (US). WU, Zheng [CN/US]; 2930 Cotton Stock Dr., Sugar Land, TX 77479 (US).	<b>(74) Agent:</b> HIGHLANDER, Steven, L.; Fulbright & Jaworski L.L.P., Suite 2400, 600 Congress Avenue, Austin, TX 78701 (US).  <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> FORMULATION OF ADENOVIRUS FOR GENE THERAPY  <b>(57) Abstract</b> <p>The present invention addresses the need to improve the long-term storage stability (i.e. infectivity) of vector formulations. In particular, it has been demonstrated that for adenovirus, the use of bulking agents, cryoprotectants and lyoprotectants imparts desired properties that allow both lyophilized and liquid adenovirus formulations to be stored at 4°C for up to 6 months and retain an infectivity between 60–100% of the starting infectivity.</p>		

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CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## FORMULATION OF ADENOVIRUS FOR GENE THERAPY

The present application claims priority to the contents of U.S. Provisional Patent Application Serial No. 60/108,606, filed November 16, 1998 and U.S. Provisional Patent  
5 Application Serial No. 60/133,116, filed May 7, 1999. The entire text of the above-referenced disclosure is specifically incorporated by reference herein without disclaimer.

**A. Field of the Invention**

The present invention relates generally to the fields of molecular biology, virus  
10 production and gene therapy. More particularly, it concerns methods for the formulation of highly purified lyophilized and liquid adenovirus particles stable for long-term storage. An important embodiment of the present invention is the use of such long-term storage virus preparations for gene therapy treatments of viral disease, genetic disease and malignancies.

15

**B. Description of Related Art**

Viruses are highly efficient at nucleic acid delivery to specific cell types, while often avoiding detection by the infected hosts immune system. These features make certain viruses attractive candidates as gene-delivery vehicles for use in gene therapies  
20 (Robbins and Ghivizzani; 1998; Cristiano *et al.*, 1998). Retrovirus, adenovirus, adeno-associated virus (AAV), and herpes simplex virus are examples of commonly used viruses in gene therapies. Each of the aforementioned viruses has its advantages and limitations, and must therefore be selected according to suitability of a given gene therapy (Robbins and Ghivizzani; 1998).

25

A variety of cancer and genetic diseases currently are being addressed by gene therapy. Cardiovascular disease (Morishita *et al.*, 1998), colorectal cancer (Fujiwara and Tanaka, 1998), lung cancer (Roth *et al.*, 1998), brain tumors (Badie *et al.*, 1998), and thyroid carcinoma (Braiden *et al.*, 1998) are examples of gene therapy treatments



currently under investigation. Further, the use of viral vectors in combination with other cancer treatments also is an avenue of current research (Jounaidi *et al.*, 1998).

Viral particles must maintain their structural integrity to be infectious and  
5 biologically active. The structural integrity of a viral vector often is compromised during the formulation process, thus precluding its use as a gene therapy vector. Adenoviruses for gene therapy traditionally have been formulated in buffers containing 10% glycerol. Formulated adenovirus is stored at  $< -60^{\circ}\text{C}$  to ensure good virus stability during storage. This ultra-low temperature storage not only is very expensive, but creates significant  
10 inconvenience for storage, transportation and clinic use. There is an urgent need to develop new formulation for adenovirus that can be stored at refrigerated condition.

Lyophilization has been used widely to improve the stability of various viral vaccine and recombinant protein products. It is expected that the long-term storage  
15 stability of adenovirus can be improved by reducing the residual water content (moisture) in the formulated product through lyophilization. However, there have not been reported studies on the lyophilization of live adenovirus for gene therapy.

Generally it is assumed that adenovirus will not maintain its infectivity when  
20 stored at refrigerated condition in a liquid form for extended period of time. As a result, there are no reported studies on formulating and storing adenovirus at refrigerated condition in a liquid form. Thus, there remains a need for long-term storage stable formulations of viral preparations.

## 25 SUMMARY OF THE INVENTION

The present invention addresses the need for improved, storage stable viral formulations, and methods for the production thereof, for use in gene therapy. In particular embodiments, a pharmaceutical adenovirus composition comprising adenovirus  
30 particles and pharmaceutical excipients, the excipients including a bulking agent and one

or more protectants, wherein the excipients are included in amounts effective to provide an adenovirus composition that is storage stable. In preferred embodiments, the adenovirus composition has an infectivity of between 60 and 100% of the starting infectivity, and a residual moisture of less than about 5%, when stored for six months at  
5 4° centigrade.

In one embodiment, the adenovirus composition is a freeze dried composition. In particular embodiments, the bulking agent in the freeze dried adenovirus composition forms crystals during freezing, wherein the bulking agent is mannitol, inositol, lactitol,  
10 xylitol, isomaltol, sorbitol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silcate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methhylcellulose or methylcellulose.

In certain embodiments, the bulking agent in the freeze dried adenovirus  
15 composition is mannitol. In other embodiments the composition is further defined as an aqueous composition comprising mannitol in a concentration of from about 1% to about 10% (w/v). In another embodiment, the aqueous composition comprises the mannitol in a concentration of from about 3% to 8%. In a preferred embodiment, the aqueous composition comprises mannitol in a concentration of from about 5% to 7%.

20

In certain embodiments, the freeze dried composition is prepared from an aqueous composition comprising a bulking agent in a concentration of from about 1% to 10% (w/v). In other embodiments the freeze dried composition is prepared from an aqueous composition comprising a bulking agent in a concentration of from about 3% to 8%. In  
25 yet other embodiments, the freeze dried composition is prepared from an aqueous composition comprising a bulking agent in a concentration of from about 5% to 7%.

In particular embodiments, pharmaceutical excipients serve as a protectants. In one embodiment, the protectant is further defined as a cryoprotectant. In certain  
30 embodiments, the cryoprotectant is a non-reducing sugar. In particularly defined

embodiments the non-reducing sugar is sucrose or trehalose. In preferred embodiments the non-reducing sugar is sucrose.

5 In certain embodiments, the composition is further defined as an aqueous composition comprising a non-reducing sugar in a concentration of from about 2% to about 10% (w/v). In other embodiments, the aqueous composition comprises the sugar in a concentration of from about 4% to 8%. In still other embodiments, the aqueous composition comprises the sugar in a concentration of from about 5% to 6%.

10 In one embodiment, the freeze dried composition is prepared from an aqueous composition comprising a non-reducing sugar in a concentration of from about 2% to 10% (w/v). In other embodiments, the freeze dried composition is prepared from an aqueous composition comprising a non-reducing sugar in a concentration of from about 4% to 8%. In yet other embodiments, the freeze dried composition is prepared from an  
15 aqueous composition comprising a non-reducing sugar in a concentration of from about 5% to 6%.

In another embodiment, the cryoprotectant is niacinamide, creatinine, monosodium glutamate, dimethyl sulfoxide or sweet whey solids.  
20

In certain embodiments, the protectant includes a lyoprotectant, wherein the lyoprotectant is human serum albumin, bovine serum albumin, PEG, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20  
25 or Tween-80. In a preferred embodiment, the lyoprotectant is human serum albumin.

In certain embodiments, the composition is further defined as an aqueous composition comprising the lyoprotectant in a concentration of from about 0.5% to about 5% (w/v). In another embodiment, the aqueous composition comprises the lyoprotectant  
30 in a concentration of from about 1% to about 4%. In still another embodiment, the

aqueous composition comprises the lyoprotectant in a concentration of from about 1% to about 3%.

5 In particular embodiments, the freeze dried composition is prepared from an aqueous composition comprising a lyoprotectant in a concentration of from about 0.5% to 5% (w/v). In other embodiments, the freeze dried composition is prepared from an aqueous composition comprising a lyoprotectant in a concentration of from about 1% to 4%. In another embodiment, the freeze dried composition is prepared from an aqueous composition comprising a lyoprotectant in a concentration of from about 1% to 3%.

10

In one embodiment, pharmaceutical excipients defined as protectants, comprise both a lyoprotectant and a cryoprotectant.

15 Also contemplated in the present invention is an aqueous pharmaceutical adenovirus composition comprising a polyol in an amount effective to promote the maintenance of adenoviral infectivity. In one embodiment, adenoviral infectivity of the adenovirus polyol composition is further defined as maintaining an infectivity of about 70% PFU/mL to about 99.9% PFU/mL of the starting infectivity when stored for six months at 4° centigrade. In preferred embodiments, adenoviral infectivity is about 80%  
20 to 95% PFU/mL of the starting infectivity when stored for six months at 4° centigrade.

In the context of the present invention, a polyol is defined as a polyhydric alcohol containing two or more hydroxyl groups. In certain embodiments, the polyol is glycerol, propylene glycol, polyethylene glycol, sorbitol or mannitol, wherein the polyol  
25 concentration is from about 5% to about 30% (w/v). In other embodiments, the polyol concentration is from about 10% to about 30%. In yet other embodiments, the polyol concentration is about 25%.

30 In a preferred embodiment, the aqueous pharmaceutical adenovirus composition comprises a polyol in an amount effective to promote the maintenance of adenoviral

infectivity, wherein the polyol is glycerol, included in a concentration of from about 5% to about 30% (w/v).

In other embodiments, the aqueous pharmaceutical adenovirus composition comprising a polyol in an amount effective to promote the maintenance of adenoviral infectivity further comprises an excipient in addition to the polyol, wherein the excipient is inositol, lactitol, xylitol, isomaltol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silicate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose, sucrose, dextrose, lactose, trehalose, glucose, maltose, niacinamide, creatinine, monosodium glutamate dimethyl sulfoxide, sweet whey solids, human serum albumin, bovine serum albumin, PEG, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 or Tween-80.

15

In further defined embodiments, the aqueous pharmaceutical adenovirus composition comprising a polyol further comprises in addition to the polyol at least a first and a second excipient, wherein the second excipient is different the first excipient, and the excipient is inositol, lactitol, xylitol, isomaltol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silicate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose, sucrose, dextrose, lactose, trehalose, glucose, maltose, niacinamide, creatinine, monosodium glutamate dimethyl sulfoxide, sweet whey solids, human serum albumin, bovine serum albumin, PEG, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 or Tween-80.

20

25

In another embodiment of the present invention, a method for the preparation of a long-term, storage stable adenovirus formulation, comprising the steps of providing adenovirus and combining the adenovirus with a solution comprising a buffer, a bulking

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agent, a cryoprotectant and a lyoprotectant; and lyophilizing the solution, whereby lyophilization of the solution produces a freeze-dried cake of the adenovirus formulation that retains high infectivity and low residual moisture.

5 In particular embodiments, the bulking agent used for preparing the freeze dried adenovirus formulation is mannitol, inositol, lactitol, xylitol, isomaltol, sorbitol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silcate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methhylcellulose or methylcellulose. In preferred embodiments, the bulking agent is mannitol, wherein  
10 mannitol comprises about 0.5% to about 8% (w/v) of the formulation.

In other embodiments, the cryoprotectant used for preparing the freeze dried adenovirus formulation is sucrose, dextrose, lactose, trehalose, glucose, maltose, niacinamide, creatinine, monosodium glutamate dimethyl sulfoxide or sweet whey solids.  
15 In preferred embodiments, the cryoprotectant is sucrose, wherein sucrose comprises about 2.5% to about 10% (w/v) of said formulation.

In further embodiments, the lyoprotectant used for preparing the freeze dried adenovirus formulation is human serum albumin, bovine serum albumin, PEG, glycine,  
20 arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 or Tween-80. In preferred embodiments, the lyoprotectant is human serum albumin.

In other embodiments, the buffer used for preparing the freeze dried adenovirus  
25 formulation is Tris-HCl, TES, HEPES, mono-Tris, brucine tetrahydrate, EPPS, tricine, or histidine, wherein the buffer is present in the formulation at a concentration at about 1 mM to 50 mM. In one preferred embodiment, the buffer used for preparing the freeze dried adenovirus formulation is Tris-HCl, wherein the Tris-HCl is included in a concentration of from about 1 mM to about 50 mM. In another embodiment, the Tris-  
30 HCl is included in a concentration of from about 5 mM to about 20 mM. In still other

embodiments, the freeze dried adenovirus formulation further comprises a salt selected from the group consisting of  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{Ca Cl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{NaCl}$  and  $\text{KCl}$ .

5 In one embodiment, lyophilizing the adenovirus formulation is carried out in the presence of an inert gas.

In certain embodiments, the method for preparing the freeze dried adenovirus formulation, wherein lyophilizing the solution comprises the steps of, freezing the solution, subjecting the solution to a vacuum and subjecting the solution to at least a first  
10 and a second drying cycle, whereby the second drying cycle reduces the residual moisture content of the freeze-dried cake to less than about 2%.

In another embodiment, a method for the preparation of a long-term storage, stable adenovirus liquid formulation, comprising the steps of providing adenovirus and  
15 combining the adenovirus with a solution comprising a buffer and a polyol, whereby the adenovirus liquid formulation retains high infectivity.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood however, that  
20 the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

25

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the  
30 detailed description of specific embodiments presented herein.

**FIG. 1.** Lyophilization Cycle of Adenovirus.

**FIG. 2.** Residual Moisture of Lyophilized Adenovirus After Secondary Drying at  
5 10°C.

**FIG. 3.** Stability of Lyophilized Adenovirus after Secondary Drying at 10°C.

**FIG. 4.** Residual Moisture of Lyophilized Adenovirus After Secondary Drying at  
10 30°C.

**FIG. 5.** Stability of Lyophilized Adenovirus after Secondary Drying at 30°C.

**FIG. 6.** HPLC Analysis of Lyophilized Adenovirus Stored at Room Temperature.  
15

**FIG 7.** HPLC Analysis of Lyophilized Adenovirus Stored at 4°C.

**FIG 8.** HPLC Analysis of Lyophilized Adenovirus Stored at -20°C.

**FIG. 9A and FIG. 9B.** Addition of DMSO to the formulation for an adenoviral  
20 vector increases the transduction efficiency. Human NSCLC xenografts were established  
on the flanks of nude mice. Animals received intratumoral injection of  $2 \times 10^{10}$  viral  
particles (vp) of Ad- $\beta$ gal formulated in either PBS + glycerol (FIG. 9A and FIG. 9B, top  
panels) or in PBS + glycerol + 5% DMSO (FIG. 9A and FIG. 9B, lower panels). Tumors  
25 were excised at either 24 (FIG. 9A) or 48 hours (FIG. 9B) post-injection and sectioned for  
histochemical analysis of reporter gene expression. Histochemical analysis was done on  
multiple sections from the tumor block to analyze vector transduction and distribution.  
Two sections for each formulation are illustrated: one from the tumor periphery (FIG. 9A  
and FIG. 9B, left panels) and one from the center of the tumor (FIG. 9A and FIG. 9B,  
30 right panels). In each section both transduction (as indicated by intensity of blue staining)



and distribution (as indicated by extent of blue staining) were improved by addition of DMSO to the formulation.

### Description of Illustrative Embodiments

5

The need for long-term stable virus formulations that can be stored at or above refrigerated temperatures without losing infectivity is highly desirable. Traditional methods of ultra-low temperature storage ( $\leq 60^{\circ}\text{C}$ ) of virus preparations often limit the storage, transportation and clinical applications of viruses. The inventors have developed optimal lyophilization formulations for freeze-drying adenovirus in which the freeze-dried products maintain their stability (*i.e.*, infectivity of 60-100% of the starting infectivity) and have a residual moisture of less than about 5% when stored for 6 months at  $4^{\circ}\text{C}$ .

15 In another embodiment, the inventors have developed long-term stable adenovirus formulations for storing adenovirus at  $4^{\circ}\text{C}$  in a liquid form that maintains stability (*i.e.*, infectivity of 60-100% of the starting infectivity) for at least 6 months.

#### A. Purification Techniques

20 A large scale process for the production and purification of adenovirus is described in U.S. Serial No. 08/975,519 filed November 20, 1997 (specifically incorporated herein by reference without disclaimer). This production process offers not only scalability and validatability but also virus purity comparable to that achieved using CsCl gradient ultracentrifugation. This process involves the preparation of recombinant adenovirus particles, the process comprising preparing a culture of producer cells by seeding producer cells into a culture medium, infecting cells in the culture after mid-log phase growth with a recombinant adenovirus comprising a selected recombinant gene operably linked to a promoter, harvesting recombinant adenovirus particles from the cell culture and removing contaminating nucleic acids. An important aspect of this process is the removal of contaminating nucleic acids using nucleases. Exemplary nucleases

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include Benzonase<sup>®</sup>, Pulmozyme<sup>®</sup>; or any other DNase or RNase commonly used within the art.

Enzymes such as Benzonase<sup>®</sup> degrade nucleic acid and have no proteolytic activity. The ability of Benzonase<sup>®</sup> to rapidly hydrolyze nucleic acids makes the enzyme ideal for reducing cell lysate viscosity. It is well known that nucleic acids may adhere to cell derived particles such as viruses. The adhesion may interfere with separation due to agglomeration, change in size of the particle or change in particle charge, resulting in little if any product being recovered with a given purification scheme. Benzonase<sup>®</sup> is well suited for reducing the nucleic acid load during purification, thus eliminating the interference and improving yield.

As with all endonuclease, Benzonase<sup>®</sup> hydrolyzes internal phosphodiester bonds between specific nucleotides. Upon complete digestion, all free nucleic acids present in solution are reduced to oligonucleotides 2 to 4 bases in length.

The present invention further employs a number of different purification techniques to purify viral vectors of the present invention. Such techniques include those based on sedimentation and chromatography and are described in more detail herein below.

### 1. Density Gradient Centrifugation

There are two methods of density gradient centrifugation, the *rate zonal technique* and the *isopycnic (equal density) technique*, and both can be used when the quantitative separation of all the components of a mixture of particles is required. They are also used for the determination of buoyant densities and for the estimation of sedimentation coefficients.

Particle separation by the rate zonal technique is based upon differences in size or sedimentation rates. The technique involves carefully layering a sample solution on top

of a performed liquid density gradient, the highest density of which exceeds that of the densest particles to be separated. The sample is then centrifuged until the desired degree of separation is effected, *i.e.*, for sufficient time for the particles to travel through the gradient to form discrete zones or bands which are spaced according to the relative  
5 velocities of the particles. Since the technique is time dependent, centrifugation must be terminated before any of the separated zones pellet at the bottom of the tube. The method has been used for the separation of enzymes, hormones, RNA-DNA hybrids, ribosomal subunits, subcellular organelles, for the analysis of size distribution of samples of polysomes and for lipoprotein fractionations.

10

The sample is layered on top of a continuous density gradient which spans the whole range of the particle densities which are to be separated. The maximum density of the gradient, therefore, must always exceed the density of the most dense particle. During centrifugation, sedimentation of the particles occurs until the buoyant density of the  
15 particle and the density of the gradient are equal (*i.e.*, where  $p_p = p_m$  in equation 2.12). At this point no further sedimentation occurs, irrespective of how long centrifugation continues, because the particles are floating on a cushion of material that has a density greater than their own.

20

Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method, the particles banding to form zones each at their own characteristic buoyant density. In cases where, perhaps, not all the components in a mixture of particles are required, a gradient range can be selected in which unwanted components of the mixture will sediment to the bottom of the centrifuge tube whilst the particles of interest sediment  
25 to their respective isopycnic positions. Such a technique involves a combination of both the rate zonal and isopycnic approaches.

Isopycnic centrifugation depends solely upon the buoyant density of the particle and not its shape or size and is independent of time. Hence soluble proteins, which have  
30 a very similar density (*e.g.*,  $p = 1.3 \text{ g cm}^{-3}$  in sucrose solution), cannot usually be

separated by this method, whereas subcellular organelles (*e.g.*, Golgi apparatus,  $p = 1.11 \text{ g cm}^{-3}$ , mitochondria,  $p = 1.19 \text{ g cm}^{-3}$  and peroxisomes,  $p = 1.23 \text{ g cm}^{-3}$  in sucrose solution) can be effectively separated.

5        As an alternative to layering the particle mixture to be separated onto a preformed gradient, the sample is initially mixed with the gradient medium to give a solution of uniform density, the gradient 'self-forming', by sedimentation equilibrium, during centrifugation. In this method (referred to as the *equilibrium isodensity method*), use is generally made of the salts of heavy metals (*e.g.*, cesium or rubidium), sucrose, colloidal  
10       silica or Metrizamide.

      The sample (*e.g.*, DNA) is mixed homogeneously with, for example, a concentrated solution of cesium chloride. Centrifugation of the concentrated cesium chloride solution results in the sedimentation of the CsCl molecules to form a  
15       concentration gradient and hence a density gradient. The sample molecules (DNA), which were initially uniformly distributed throughout the tube now either rise or sediment until they reach a region where the solution density is equal to their own buoyant density, *i.e.* their isopycnic position, where they will band to form zones. This technique suffers from the disadvantage that often very long centrifugation times (*e.g.*, 36 to 48 hours) are  
20       required to establish equilibrium. However, it is commonly used in analytical centrifugation to determine the buoyant density of a particle, the base composition of double stranded DNA and to separate linear from circular forms of DNA.

      Many of the separations can be improved by increasing the density differences  
25       between the different forms of DNA by the incorporation of heavy isotopes (*e.g.*,  $^{15}\text{N}$ ) during biosynthesis, a technique used by Leselson and Stahl to elucidate the mechanism of DNA replication in *Escherichia coli*, or by the binding of heavy metal ions or dyes such as ethidium bromide. Isopycnic gradients have also been used to separate and purify viruses and analyze human plasma lipoproteins.

## 2. Chromatography

Purification techniques are well known to those of skill in the art. These techniques tend to involve the fractionation of the cellular milieu (*e.g.*, density gradient centrifugation) to separate the adenovirus particles from other components of the mixture. Having separated adenoviral particles from the other components, the adenovirus may be purified using chromatographic and electrophoretic techniques to achieve complete purification. Analytical methods particularly suited to the preparation of a pure adenoviral particle of the present invention are ion-exchange chromatography, size exclusion chromatography and polyacrylamide gel electrophoresis. A particularly efficient purification method to be employed in conjunction with the present invention is HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an adenoviral particle. The term "purified" as used herein, is intended to refer to a composition, isolatable from other components, wherein the adenoviral particle is purified to any degree relative to its naturally-obtainable form. A purified adenoviral particle therefore also refers to an adenoviral component, free from the environment in which it may naturally occur.

Generally, "purified" will refer to an adenoviral particle that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the particle, protein or peptide forms the major component of the composition, such as constituting about 50% or more of the constituents in the composition.

Various methods for quantifying the degree of purification of a protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for

assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number". The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

There is no general requirement that the adenovirus, always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

Of course, it is understood that the chromatographic techniques and other purification techniques known to those of skill in the art may also be employed to purify proteins expressed by the adenoviral vectors of the present invention. Ion exchange chromatography and high performance liquid chromatography are exemplary purification techniques employed in the purification of adenoviral particles and are described in further detail herein below.

25

#### a. Ion-Exchange Chromatography

The basic principle of ion-exchange chromatography is that the affinity of a substance for the exchanger depends on both the electrical properties of the material and the relative affinity of other charged substances in the solvent. Hence, bound material can be eluted by changing the pH, thus altering the charge of the material, or by adding

30

competing materials, of which salts are but one example. Because different substances have different electrical properties, the conditions for release vary with each bound molecular species. In general, to get good separation, the methods of choice are either continuous ionic strength gradient elution or stepwise elution. (A gradient of pH alone is not often used because it is difficult to set up a pH gradient without simultaneously increasing ionic strength.) For an anion exchanger, either pH and ionic strength are gradually increased or ionic strength alone is increased. For a cation exchanger, both pH and ionic strength are increased. The actual choice of the elution procedure is usually a result of trial and error and of considerations of stability. For example, for unstable materials, it is best to maintain fairly constant pH.

An ion exchanger is a solid that has chemically bound charged groups to which ions are electrostatically bound; it can exchange these ions for ions in aqueous solution. Ion exchangers can be used in column chromatography to separate molecules according to charge; actually other features of the molecule are usually important so that the chromatographic behavior is sensitive to the charge density, charge distribution, and the size of the molecule.

The principle of ion-exchange chromatography is that charged molecules adsorb to ion exchangers reversibly so that molecules can be bound or eluted by changing the ionic environment. Separation on ion exchangers is usually accomplished in two stages: first, the substances to be separated are bound to the exchanger, using conditions that give stable and tight binding; then the column is eluted with buffers of different pH, ionic strength, or composition and the components of the buffer compete with the bound material for the binding sites.

An ion exchanger is usually a three-dimensional network or matrix that contains covalently linked charged groups. If a group is negatively charged, it will exchange positive ions and is a cation exchanger. A typical group used in cation exchangers is the sulfonic group,  $\text{SO}_3^-$ . If an  $\text{H}^+$  is bound to the group, the exchanger is said to be in the

acid form; it can, for example, exchange on  $H^+$  for one  $Na^+$  or two  $H^+$  for one  $Ca^{2+}$ . The sulfonic acid group is called a strongly acidic cation exchanger. Other commonly used groups are phenolic hydroxyl and carboxyl, both weakly acidic cation exchangers. If the charged group is positive - for example, a quaternary amino group--it is a strongly basic anion exchanger. The most common weakly basic anion exchangers are aromatic or aliphatic amino groups.

The matrix can be made of various material. Commonly used materials are dextran, cellulose, agarose and copolymers of styrene and vinylbenzene in which the divinylbenzene both cross-links the polystyrene strands and contains the charged groups. Table 1 gives the composition of many ion exchangers.

The total capacity of an ion exchanger measures its ability to take up exchangeable groups per milligram of dry weight. This number is supplied by the manufacturer and is important because, if the capacity is exceeded, ions will pass through the column without binding.



TABLE 1

Matrix	Exchanger	Functional Group	Tradename
Dextran	Strong Cationic	Sulfopropyl	SP-Sephadex
	Weak Cationic	Carboxymethyl	CM-Sephadex
	Strong Anionic	Diethyl-(2-hydroxypropyl)-aminoethyl	QAE-Sephadex
	Weak Anionic	Diethylaminoethyl	DEAE-Sephadex
Cellulose	Cationic	Carboxymethyl	CM-Cellulose
	Cationic	Phospho	P-cel
	Anionic	Diethylaminoethyl	DEAE-cellulose
	Anionic	Polyethylenimine	PEI-Cellulose
	Anionic	Benzoylated-naphthoylated, diethylaminoethyl	DEAE(BND)-cellulose
	Anionic	p-Aminobenzyl	PAB-cellulose
Styrene-divinylbenzene	Strong Cationic	Sulfonic acid	AG 50
	Strong Anionic		AG 1
	Strong Cationic	Sulfonic acid + Tetramethylammonium	AG 501
	Strong Anionic		
Acrylic	Weak Cationic	Carboxylic	Bio-Rex 70
Phenolic	Strong Cationic	Sulfonic acid	Bio-Rex 40
Expoxyamine	Weak Anionic	Tertiary amino	AG-3

The available capacity is the capacity under particular experimental conditions (i.e., pH, ionic strength). For example, the extent to which an ion exchanger is charged depends on the pH (the effect of pH is smaller with strong ion exchangers). Another factor is ionic strength because small ions near the charged groups compete with the sample molecule for these groups. This competition is quite effective if the sample is a macromolecule because the higher diffusion coefficient of the small ion means a greater number of encounters. Clearly, as buffer concentration increases, competition becomes keener.

The porosity of the matrix is an important feature because the charged groups are both inside and outside the matrix and because the matrix also acts as a molecular sieve. Large molecules may be unable to penetrate the pores; so the capacity will decrease with increasing molecular dimensions. The porosity of the polystyrene-based resins is determined by the amount of cross-linking by the divinylbenzene (porosity decreases with increasing amounts of divinylbenzene). With the Dowex and AG series, the percentage of divinylbenzene is indicated by a number after an X - hence, Dowex 50-X8 is 8% divinylbenzene

Ion exchangers come in a variety of particle sizes, called mesh size. Finer mesh means an increased surface-to-volume ration and therefore increased capacity and decreased time for exchange to occur for a given volume of the exchanger. On the other hand, fine mesh means a slow flow rate, which can increase diffusional spreading. The use of very fine particles, approximately 10  $\mu\text{m}$  in diameter and high pressure to maintain an adequate flow is called *high-performance* or *high-pressure liquid chromatography* or simply HPLC.

Such a collection of exchangers having such different properties - charge, capacity, porosity, mesh - makes the selection of the appropriate one for accomplishing a particular separation difficult. How to decide on the type of column material and the conditions for binding and elution is described in the following Examples.

There are a number of choice to be made when employing ion exchange chromatography as a technique. The first choice to be made is whether the exchanger is to be anionic or cationic. If the materials to be bound to the column have a single charge (i.e., either plus or minus), the choice is clear. However, many substances (e.g., proteins, viruses), carry both negative and positive charges and the net charge depends on the pH. In such cases, the primary factor is the stability of the substance at various pH values. Most proteins have a pH range of stability (i.e., in which they do not denature) in which they are either positively or negatively charged. Hence, if a protein is stable at pH values above the isoelectric point, an anion exchanger should be used; if stable at values below the isoelectric point, a cation exchanger is required.

The choice between strong and weak exchangers is also based on the effect of pH on charge and stability. For example, if a weakly ionized substance that requires very low or high pH for ionization is chromatographed, a strong ion exchanger is called for because it functions over the entire pH range. However, if the substance is labile, weak ion exchangers are preferable because strong exchangers are often capable of distorting a molecule so much that the molecule denatures. The pH at which the substance is stable must, of course, be matched to the narrow range of pH in which a particular weak exchanger is charged. Weak ion exchangers are also excellent for the separation of molecules with a high charge from those with a small charge, because the weakly charged ions usually fail to bind. Weak exchangers also show greater resolution of substances if charge differences are very small. If a macromolecule has a very strong charge, it may be impossible to elute from a strong exchanger and a weak exchanger again may be preferable. In general, weak exchangers are more useful than strong exchangers.

The Sephadex and Bio-gel exchangers offer a particular advantage for macromolecules that are unstable in low ionic strength. Because the cross-links in these materials maintain the insolubility of the matrix even if the matrix is highly polar, the density of ionizable groups can be made several times greater than is possible with

cellulose ion exchangers. The increased charge density means increased affinity so that adsorption can be carried out at higher ionic strengths. On the other hand, these exchangers retain some of their molecular sieving properties so that sometimes molecular weight differences annul the distribution caused by the charge differences; the molecular sieving effect may also enhance the separation.

Small molecules are best separated on matrices with small pore size (high degree of cross-linking) because the available capacity is large, whereas macromolecules need large pore size. However, except for the Sephadex type, most ion exchangers do not afford the opportunity for matching the porosity with the molecular weight.

The cellulose ion exchangers have proved to be the best for purifying large molecules such as proteins and polynucleotides. This is because the matrix is fibrous, and hence all functional groups are on the surface and available to even the largest molecules. In many cases however, beaded forms such as DEAE-Sephacel and DEAE-Biogel P are more useful because there is a better flow rate and the molecular sieving effect aids in separation.

Selecting a mesh size is always difficult. Small mesh size improves resolution but decreases flow rate, which increases zone spreading and decreases resolution. Hence, the appropriate mesh size is usually determined empirically.

Because buffers themselves consist of ions, they can also exchange, and the pH equilibrium can be affected. To avoid these problems, the *rule of buffers* is adopted: use *cationic buffers with anion exchangers* and *anionic buffers with cation exchangers*. Because ionic strength is a factor in binding, a buffer should be chosen that has a high buffering capacity so that its ionic strength need not be too high. Furthermore, for best resolution, it has been generally found that the ionic conditions used to apply the sample to the column (the so-called *starting conditions*) should be near those used for eluting the column.

**b. High Performance Liquid Chromatography**

High performance liquid chromatography(HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

**B. Viral Formulation**

Retrovirus, adenovirus, adeno-associated virus, and herpes simplex virus are the most commonly used viruses in gene therapy (Robbins and Ghivizzani; 1998). It is contemplated in the present invention that the preparation of long-term stable adenovirus vectors that can be stored at or above refrigerated temperatures would be useful as gene therapy vectors. Viral particles must maintain their structural integrity to remain infective and biologically active for use as gene therapy vectors. Current virus formulations do not readily make it feasible to store or transport viral vector at or above refrigerated temperatures without significant loss of viral infectivity.

The present invention describes long-term stable adenovirus formulations that can be stored at 4°C for periods up to 6 months. In one embodiment of the present invention, adenovirus preparations are formulated for lyophilization and long-term storage at 4°C as freeze-dried adenovirus. In another embodiment, the adenovirus is prepared as a liquid formulation that is long-term stable at 4°C. An important aspect of both the lyophilized and liquid adenovirus formulations is the addition of at least one or more compounds that improve the long-term, storage stability of the adenovirus.

The term "compound" in the context of the present invention includes pharmaceutically acceptable carriers such as bulking agents, cryoprotectants, lyoprotectants, preservatives, solvents, solutes and any additional pharmaceutical agents well known in the art. Buffering agents and other types of pH control can also be added  
5 simultaneously in order to provide for maximum buffering capacity for the adenovirus formulation. For example, pH changes that deviate from physiological conditions often result in irreversible aggregation of proteins (Wetzel, 1992) and viral capsids (Misselwitz *et al.*, 1995) due to complete or partial denaturation of the protein. Thus, buffering agents are particularly important for virus preparations that aggregate or denature at sub-optimal  
10 pH ranges.

#### 1. Lyophilized Formulations

The formulation of lyophilized, long-term storage stable adenovirus in the present invention requires the presence of one or more excipients. More particularly, for optimal  
15 long-term stability of lyophilized adenovirus formulations, a bulking agent and one or more protectants are desirable. It is well known in the art that loss in virus infectivity often is directly related to denaturation, self association and aggregation of the viral particles (Misselwitz *et al.*, 1995; Vanlandschoot *et al.*, 1998; Sagrera *et al.*, 1998; Lu *et al.*, 1998). In fact, the *E. coli* heat shock proteins GroEL/GroES have been shown to both  
20 stabilize viral particles from denaturation and aggregation during high stress cellular conditions and to facilitate capsid assembly during non-stressed, normal cellular conditions (Polissi *et al.*, 1995; Nakonechny and Teschke, 1998).

The use of bulking agents, cryoprotectants, lyoprotectants and salts in the present  
25 invention are included in the formulation of lyophilized adenovirus to improve long-term stability (*i.e.* infectivity) of the adenovirus freeze-dried products. The stabilizing effect of the cryoprotectant sucrose against irreversible denaturation and aggregation has been described previously as an excluded volume effect (Hall *et al.*, 1995). Similarly, bulking agents, cryo- and lyoprotectants such as polyacrylamide gels, agarose gels, dextran and  
30 polyethylene glycol (PEG) have demonstrated enhanced stabilities of proteins and nucleic

acids in part by excluded volume effects (Fried and Bromberg, 1997; Vossen and Fried, 1997). The exact mechanistic details of excluded volume effects are still not clear. A currently accepted theory is that many of these compounds result in the preferential hydration of protein molecules (*i.e.* volume of exclusion), which tends to stabilize the native versus the denatured conformation of proteins, and therefore prevents aggregation. In addition, the presence of low concentrations of cosolvents (*e.g.*, salts) result in charge screening of proteins and viral protein coats increasing their solubility in water.

The use of bulking agents, cryoprotectants, lyoprotectants and salts in the present invention are contemplated and demonstrated experimentally to improve the storage stability of lyophilized adenovirus products. In one embodiment, a bulking agent and protectants are combined with a buffer comprising adenovirus.

Bulking agents, cryoprotectants and lyoprotectants are well known in the art (Lueckel *et al.*, 1998; Herman *et al.*, 1994; Croyle *et al.*, 1998; Corveleyn and Remon, 1996). Bulking agents considered in the present invention are mannitol, inositol, lactitol, xylitol, isomaltol, sorbitol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silcate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methhylcellulose, methylcellulose and other bulking agents well known in the art. Cryoprotectants considered are sucrose, dextrose, lactose, trehalose, glucose, maltose, niacinamide, creatinine, monosodium glutamate, dimethyl sulfoxide, sweet whey solids, as well as other known cryoprotectants. Lyoprotectants contemplated for use in the present invention are human serum albumin, bovine serum albumin, PEG, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 and Tween-80. Certain lyoprotectants are also classified as cryoprotectants and vice versa. For the purpose of the present invention, cryoprotectants and lyoprotectants are represented as independent classes of compounds. However, this classification is only for clarity of the invention and should not limit the person skilled in the art from using any excipient that stabilizes the adenovirus formulation. In other embodiments of the present

invention, the term excipient encompasses bulking agents, cryo- and lyoprotectants. In certain embodiments, salts are included in the formulation in addition to the aforementioned excipients. The following salts are considered for use in the present invention  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{CaCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{NaCl}$ , and  $\text{KCl}$ , but should not preclude the use of other salts that improve stability of the adenovirus formulation.

In other embodiments of the invention, the lyophilized adenovirus formulation is dried in the presence of an inert gas or a combination of inert gasses. The purging of the lyophilization vessel with an inert gas or gasses, the presence of the inert gas or gasses during lyophilization of the adenovirus solution and during the capping of the lyophilization vial after the drying step, are contemplated to minimize the deleterious effects of  $\text{O}_2$ . It is known that residual  $\text{O}_2$  leads to oxidation and degradation of proteins. It is contemplated that purging and capping of the freeze-dried adenovirus product improves the long-term storage stability of the adenovirus product. The use of antioxidants such as  $\beta$ -mercapto ethanol, DTT, citric acid and the like may also be considered for use in formulations.

An important aspect of the lyophilization process is a second drying cycle. The second drying cycle is at a temperature of  $30^\circ\text{C}$  for at least 3.5 hours, which is demonstrated to reduce the residual moisture of the adenovirus freeze-dried product to less than 2% water immediately after drying. It is contemplated that the reduced residual moisture improves the long-term storage stability of the adenovirus freeze-dried product. Longer drying times up to 20 hours are thus contemplated to further reduce residual moisture.

25

## 2. Liquid Formulations

The formulation of liquid, long-term storage stable adenovirus in the present invention requires the presence of a polyol. A polyol is a polyhydric alcohol containing two or more hydroxyl groups. For optimal long-term stability of liquid adenovirus formulations in the present invention, glycerol is used. In particular embodiments of the

30



invention, the presence 20% glycerol results in adenovirus stability (80% PFU/mL) for periods of time at least up to 6 months days when stored at 4°C.

5 Glycerol (glycerin) is one of the oldest and most widely used excipients in pharmaceutical products. It is a clear, colorless liquid which is miscible with water and alcohol. Glycerol is hygroscopic, stable to mild acidic and basic environments and can be sterilized at temperatures up to 150°C. It is well known as both a taste masking and cryoprotective agent, as well as an antimicrobial agent. It has good solubilizing power and is a commonly used solvent in parenteral formulations. It is considered to be one of  
10 the safest excipients used since it is metabolized to glucose, or to substances which are involved with triglyceride synthesis or glycolysis (Frank *et al.*, 1981). It is a GRAS listed excipient and typically used at levels up to 50% in parenteral formulations.

The stabilizing effects of glycerol on protein structure is well known in the art  
15 (Hase *et al.*, 1998; Juranville *et al.*, 1998). Several studies indicate that glycerol has a similar effect of viral particles. For example, when competent Haemophilus influenza bacteria were exposed to purified phage and plated for transfectants, a 100-fold increase in transfectants was observed when 32% glycerol was present in the solution (Stuy, 1986). In yet another study, glycerol was demonstrated to preserve the integrity of vaccinia virus  
20 (Slonin and Roslerova, 1969).

Other polyols contemplated for use in the present invention are polyethylene glycol, propylene glycol, sorbitol, mannitol, and the like. Polyethylene glycols are polymers of ethylene oxide with the general formula:



where n represents the number of oxyethylene groups. The PEG's are designated by a numerical value, which is indicative of the average molecular weight for a given grade. Molecular weights below 600 are liquids, and molecular weights above 1000 are solids at room temperature. These polymers are readily soluble in water, which make  
30 them quite useful for parenteral dosage forms. Only PEG 400 and PEG 300 are utilized

in parenteral products, typically at concentrations up to 30% v/v. These polymers are generally regarded as non-toxic and non-irritating. There are numerous reviews regarding the pharmaceutical and toxicological characteristics of these polyols (Smyth *et al.*, 1950; Rowe and Wolf, 1982, Swarbrick and Boylan, 1990).

5

Propylene glycol, a dihydroxy alcohol, is one of the more common solvents encountered in pharmaceutical cosolvent formulations, for both parenteral and non-parenteral dosage forms. PG is generally regarded as non-toxic. It is more hygroscopic than glycerin, and has excellent solubilizing power for a wide variety of compounds. In addition, it has excellent bacteriocidal and preservative properties (Heine *et al.*, 1950). PG is metabolized to carbon dioxide and water *via* lactic and pyruvic acid intermediates and, therefore, not prone to the severe toxicities.

Sorbitol and mannitol are hexahydric alcohols, consisting of white, crystalline powders, that are soluble in water. Both are commonly used excipients in pharmaceutical products with little or no toxicity associated, as approved by the FDA for food use. The mechanistics of sorbitol and mannitol protein and viral stabilization is still not completely understood. Current theories suggest at least part of the effect is osmotic diuretic (Vanholder, *et al.*, 1984; de Rizzo, *et al.*, 1988). The use of the polyols described above are considered exemplary, but should not limit the skilled artist from selecting other polyols that confer viral stability for liquid formulations.

It is also contemplated, in addition to a polyol in the liquid formulation, that one or possibly two excipients may also be included. Excipients considered for use in the present invention are inositol, lactitol, xylitol, isomaltol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silcate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methhylcellulose, methylcellulose, sucrose, dextrose, lactose, trehalose, glucose, maltose, niacinamide, creatinine, monosodium glutamate, dimethyl sulfoxide, sweet whey solids, human serum albumin, bovine serum albumin, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine,

polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 and Tween-80. The choice of a particular excipient is dependent in some instances on the desired properties of the viral formulation.

5           In particular embodiments, dimethyl sulfoxide (DMSO) is contemplated for use in the present invention. DMSO has been demonstrated to enhance the infectivity of adenovirus preparations by increasing the efficiency of gene transfer (Chikada and Jones, 1999). For example, the infectivity of adenovirus type 2 DNA in 293 cells was increased up to five-fold by the brief treatment of cell monolayers with 25% DMSO (Chinnadurai *et*  
10 *al.*, 1978). The stabilization of virus particles *via* DMSO also has been reported (Wallis and Melnick, 1968). The present inventors demonstrate that the intratumoral administration of Ad-p53 is improved when DMSO is added to 5 or 10% (see FIG. 9). Adenovirus studies *via* intravesical administration indicate that an adenoviral vector may be stable in up to 50% DMSO (WO 98/35554). In other embodiments, a polyol  
15 contemplated for use in the present invention as an enhancer of adenovirus gene transduction is a polyoxyalkene (U. S. Patent 5,552,309, specifically incorporate herein by reference in its entirety).

          Thus in particular embodiments, an adenoviral formulation according to the  
20 present invention may also contain DMSO. The concentration for intratumoral administration may contain from about 2% to 67% DMSO, preferably from about 5% to 20%. The concentration for intravesical administration may contain from about 2% to 67% DMSO, preferably from about 20% to 50%. The concentration for topical administration may contain from about 2% to 67% DMSO, preferably from about 10% to  
25 40%. The concentration for intra-articular administration may contain from about 2% to 67% DMSO, preferably, from about 5% to 40%. The concentration for systemic administration may contain from about 2% to 75% DMSO, preferably from about 50% to 67%.

Adenovirus polyol formulations of the invention may future comprise a polyoxamer, such as Polyoxamer 407, at concentrations of from about 0.5% to 20%, preferably from about 10% to 20%. The formulation storage stable adenovirus may also contain from about 5% to 40% dimethylacetamide, preferably from about 10% to 25%,  
5 Or it may contain from about 10% to 50% of a polyethylene glycol, such as polyethylene glycol 400, preferably from about 15% to 50%. Of course, the formulation of said adenovirus also may contain combinations of the above components.

### C. Viral Transformation

10 The present invention employs, in one example, adenoviral infection of cells in order to generate therapeutically significant vectors. Typically, the virus will simply be exposed to the appropriate host cell under physiologic conditions, permitting uptake of the virus. Though adenovirus is exemplified, the present methods may be advantageously employed with other viral vectors, as discussed below.

15

#### 1. Viral Infection

##### a. Adenovirus

One method for delivery of the recombinant DNA involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low  
20 capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a recombinant gene construct that has been cloned therein.

25

The vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host  
30 cells does not result in chromosomal integration because adenoviral DNA can replicate in

an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its  
5 mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes  
10 proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant  
15 processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

20 In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

25

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is  
30 dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus

vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) have disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be

crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*,  $10^9$ - $10^{11}$  plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Adenoviral vectors also have been described for treatment of certain types of cancers (U.S. Patent 5,789,244, specifically incorporated herein by

reference in its entirety). Animal studies have suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; 5 Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

#### b. Retrovirus

10 The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its 15 descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also 20 required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing 25 the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are 30 then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann



*et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

5

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

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### c. Adeno-Associated Virus

Adeno-associated virus (AAV) is an attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture (Muzyczka, 1992). AAV has a broad host range for infectivity (Tratschin, *et al.*, 1984; Laughlin, *et al.*, 1986; Lebkowski, *et al.*, 1988; McLaughlin, *et al.*, 1988), which means it is applicable for use with the present invention. Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference.

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Studies demonstrating the use of AAV in gene delivery include LaFace *et al.* (1988); Zhou *et al.* (1993); Flotte *et al.* (1993); and Walsh *et al.* (1994). Recombinant AAV vectors have been used successfully for *in vitro* and *in vivo* transduction of marker genes (Kaplitt *et al.*, 1994; Lebkowski *et al.*, 1988; Samulski *et al.*, 1989; Shelling and Smith, 1994; Yoder *et al.*, 1994; Zhou *et al.*, 1994; Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1985; McLaughlin *et al.*, 1988) and genes involved in human diseases (Flotte *et al.*, 1992; Luo *et al.*, 1994; Ohi *et al.*, 1990; Walsh *et al.*, 1994; Wei *et al.*,

25

1994). Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

AAV is a dependent parvovirus in that it requires coinfection with another virus  
5 (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells (Muzyczka, 1992). In the absence of coinfection with helper virus, the wild-type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus (Kotin *et al.*, 1990; Samulski *et al.*, 1991). rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep  
10 protein is also expressed (Shelling and Smith, 1994). When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is established (Samulski *et al.*, 1989; McLaughlin *et al.*, 1988; Kotin *et al.*, 1990; Muzyczka, 1992).

15

Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid containing the gene of interest flanked by the two AAV terminal repeats (McLaughlin *et al.*, 1988; Samulski *et al.*, 1989; each incorporated herein by reference) and an expression plasmid containing the wild-type AAV coding sequences without the terminal  
20 repeats, for example pIM45 (McCarty *et al.*, 1991; incorporated herein by reference). The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the rAAV particles (for example, by cesium chloride density centrifugation). Alternatively,  
25 adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used (Yang *et al.*, 1994a; Clark *et al.*, 1995). Cell lines carrying the rAAV DNA as an integrated provirus can also be used (Flotte *et al.*, 1995).

#### d. Other Viral Vectors

Other viral vectors may be employed as constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) and herpesviruses may be employed. They offer  
5 several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro*  
10 studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface,  
15 and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

20 Also contemplated for use in the present invention is a fairly new class of viruses termed oncolytic virus (Pennisi, 1998). Some of the viruses included in this group are reovirus, the genetically modified adenovirus OYNX-015 and CN706. These oncolytic viruses, which have not been genetically altered to prevent their replication, destroy certain types of cancer cells by multiplying and spreading, killing only the cancer cells.  
25 Each of the above oncolytic viruses are proposed to operate via different pathways involved in cancers.

For example, human reovirus requires an activated Ras signaling pathway for infection of cultured cells. Thus, in certain tumors with an overactive *ras* gene, reovirus  
30 readily replicates. In a study on reovirus, severe combined immune deficient mice

bearing tumors established from *v-erbB*-transformed murine NIH 3T3 cells or human U87 glioblastoma cells were treated with the virus. A single intratumoral injection of virus resulted in regression of tumors in 65% to 80 % of the mice. Treatment of immune-competent C3H mice bearing tumors established from a *ras*-transformed C3H-10T1/2 cells also resulted in tumor regression, although a series of injections were required (Coffey *et al.*, 1998)

## 2. Vectors and Regulatory Signals

Vectors of the present invention are designed, primarily, to transform cells with a gene under the control of regulated eukaryotic promoters (*i.e.*, inducible, repressable, tissue specific). Also, the vectors usually will contain a selectable marker if, for no other reason, to facilitate their production *in vitro*. However, selectable markers may play an important role in producing recombinant cells and thus a discussion of promoters is useful here. Table 2 and Table 3 below, list inducible promoter elements and enhancer elements, respectively.

**Table 2 - Inducible Elements**

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger and Karin, 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987; Karin @, 1987; Angel <i>et al.</i> , 1987b; McNeall <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors and Varmus, 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Fonta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1986
$\beta$ -Interferon	poly(rI)X poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 <u>E2</u>	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angle <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angle <i>et al.</i> , 1987b

Element	Inducer	References
SV40	Phorbol Ester (TFA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
$\alpha$ -2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2kb	Interferon	Blanar <i>et al.</i> , 1989
HSP70	Ela, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone $\alpha$ Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

**Table 3 - Other Promoter/Enhancer Elements**

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Hanerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1988; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> , 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987, Winoto and Baltimore, 1989; Redondo <i>et al.</i> , 1990
HLA DQ $\alpha$ and DQ $\beta$	Sullivan and Peterlin, 1987
$\beta$ -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn and Maniatis, 1985
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DR $\alpha$	Sherman <i>et al.</i> , 1989
$\beta$ -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> , 1989
Muscle Creatine Kinase	Jaynes <i>et al.</i> , 1988; Horlick and Benfield, 1989; Johnson <i>et al.</i> , 1989a
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Omitz <i>et al.</i> , 1987
Metallothionein	Karin <i>et al.</i> , 1987; Culotta and Hamer, 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987
Albumin Gene	Pinkert <i>et al.</i> , 1987, Tronche <i>et al.</i> , 1989, 1990
$\alpha$ -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
$\beta$ -Globin	Trudel and Constantini, 1987
e-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985

Promoter/Enhancer	References
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch <i>et al.</i> , 1990
$\alpha$ 1-Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990
SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleight and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; deVilliers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a,b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander and Haseltine, 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Chol <i>et al.</i> , 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky and Botchan, 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens and Hentschel, 1987; Glue <i>et al.</i> , 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986;

Promoter/Enhancer	References
	Shaul and Ben-Levy, 1987; Spandau and Lee, 1988
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber and Cullan, 1988; Jakobovits <i>et al.</i> , 1988; Feng and Holland, 1988; Takebe <i>et al.</i> , 1988; Rowen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp and Marciniak, 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

Another signal that may prove useful is a polyadenylation signal (hGH, BGH, SV40).

- 5           The use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5'-methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and
- 10           Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently
- 15           expressed using a single promoter/enhancer to transcribe a single message.

- As discussed above, in certain embodiments of the invention, a cell may be identified and selected *in vitro* or *in vivo* by including a marker in the expression construct. Such markers confer an identifiable change to the cell permitting easy
- 20           identification of cells containing the expression construct. Usually, the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin,



tetracycline and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed.

5           The promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

10

          The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early  
15 transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins.

20

          At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

25

          Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that  
30 promoter function is preserved when elements are inverted or moved relative to one

another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

5        Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of  
10       many individual elements, each of which binds to one or more transcriptional proteins.

      The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a  
15       promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

      Promoters and enhancers have the same general function of activating  
20       transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

25

      In any event, it will be understood that promoters are DNA elements which when positioned functionally upstream of a gene leads to the expression of that gene. Most transgene constructs of the present invention are functionally positioned downstream of a promoter element.

30

#### D. Engineering of Viral Vectors

In certain embodiments, the present invention further involves the manipulation of viral vectors. Such methods involve the use of a vector construct containing, for example, a heterologous DNA encoding a gene of interest and a means for its expression, replicating the vector in an appropriate helper cell, obtaining viral particles produced therefrom, and infecting cells with the recombinant virus particles. The gene could simply encode a protein for which large quantities of the protein are desired, *i.e.*, large scale *in vitro* production methods. Alternatively, the gene could be a therapeutic gene, for example to treat cancer cells, to express immunomodulatory genes to fight viral infections, or to replace a gene's function as a result of a genetic defect. In the context of the gene therapy vector, the gene will be a heterologous DNA, meant to include DNA derived from a source other than the viral genome which provides the backbone of the vector. Finally, the virus may act as a live viral vaccine and express an antigen of interest for the production of antibodies they are against. The gene may be derived from a prokaryotic or eukaryotic source such as a bacterium, a virus, a yeast, a parasite, a plant, or even an animal. The heterologous DNA also may be derived from more than one source, *i.e.*, a multigene construct or a fusion protein. The heterologous DNA may also include a regulatory sequence which may be derived from one source and the gene from a different source.

##### 1. Therapeutic Genes

p53 currently is recognized as a tumor suppressor gene (Montenarh, 1992). High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses, including SV40. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently-mutated gene in common human cancers (Mercer, 1992). It is mutated in over 50% of human NSCLC (Hollestein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino-acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are generally minute by comparison with transformed cells or tumor tissue. Interestingly, wild-type p53 appears to be important in regulating cell growth and division. Overexpression of wild-type p53 has been shown in some cases to be anti-proliferative in human tumor cell lines. Thus, p53 can act as a negative regulator of cell growth (Weinberg, 1991) and may directly suppress uncontrolled cell growth or directly or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild-type p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene.

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Casey and colleagues have reported that transfection of DNA encoding wild-type p53 into two human breast cancer cell lines restores growth suppression control in such cells (Casey *et al.*, 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, p53 into human lung cancer cell lines (Takahasi *et al.*, 1992). p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 is not detrimental to normal cells with endogenous wild-type p53. Thus, such constructs might be taken up by normal cells without adverse effects. It is thus proposed that the treatment of p53-associated cancers with wild-type p53 expression constructs will reduce the number of malignant cells or their growth rate. Furthermore, recent studies suggest

that some p53 wild-type tumors are also sensitive to the effects of exogenous p53 expression.

The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G<sub>1</sub> phase. The activity of this enzyme may be to phosphorylate Rb at late G<sub>1</sub>. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, *e.g.* p16<sup>INK4</sup>, which has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since the p16<sup>INK4</sup> protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

p16<sup>INK4</sup> belongs to a newly described class of CDK-inhibitory proteins that also includes p16<sup>B</sup>, p21<sup>WAF1</sup>, CIP1, SDI1, and p27<sup>KIP1</sup>. The p16<sup>INK4</sup> gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the p16<sup>INK4</sup> gene are frequent in human tumor cell lines. This evidence suggests that the p16<sup>INK4</sup> gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the p16<sup>INK4</sup> gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994a; Kamb *et al.*, 1994b; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). Restoration of wild-type p16<sup>INK4</sup> function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995).

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that

neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung *et al.* (1993a; 1993b and 1993c) demonstrated  
5 that the first Ig domain of C-CAM is critical for cell adhesion activity.

Cell adhesion molecules, or CAMs are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (Edelman, 1985). Recent data indicate that aberrant expression of CAMs may be  
10 involved in the tumorigenesis of several neoplasms; for example, decreased expression of E-cadherin, which is predominantly expressed in epithelial cells, is associated with the progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Matura *et al.*, 1992; Umbas *et al.*, 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of  $\alpha_5\beta_1$  integrin  
15 by gene transfer can reduce tumorigenicity of Chinese hamster ovary cells *in vivo*. C-CAM now has been shown to suppress tumor growth *in vitro* and *in vivo*.

Other tumor suppressors that may be employed according to the present invention include RB, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, BRCA1, VHL,  
20 FCC, MMAC1, MCC, p16, p21, p57, C-CAM, p27 and BRCA2. Inducers of apoptosis, such as Bax, Bak, Bcl-X<sub>s</sub>, Bik, Bid, Harakiri, Ad E1B, Bad and ICE-CED3 proteases, similarly could find use according to the present invention.

Various enzyme genes are of interest according to the present invention. Such  
25 enzymes include cytosine deaminase, hypoxanthine-guanine phosphoribosyltransferase, galactose-1-phosphate uridyltransferase, phenylalanine hydroxylase, glucocerebrosidase, sphingomyelinase,  $\alpha$ -L-iduronidase, glucose-6-phosphate dehydrogenase, HSV thymidine kinase and human thymidine kinase.

Hormones are another group of gene that may be used in the vectors described herein. Included are growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I and II,  $\beta$ -endorphin,  $\beta$ -melanocyte stimulating hormone ( $\beta$ -MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins, somatostatin, calcitonin, calcitonin gene related peptide (CGRP),  $\beta$ -calcitonin gene related peptide, hypercalcemia of malignancy factor (1-40), parathyroid hormone-related protein (107-139) (PTH-rP), parathyroid hormone-related protein (107-111) (PTH-rP), glucagon-like peptide (GLP-1),  
5 pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalinamide, metorphinamide, alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5-28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P  
10 and thyrotropin releasing hormone (TRH).

Other classes of genes that are contemplated to be inserted into the vectors of the present invention include interleukins and cytokines. Interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF and G-CSF.  
20

Examples of diseases for which the present viral vector would be useful include, but are not limited to, adenosine deaminase deficiency, human blood clotting factor IX deficiency in hemophilia B, and cystic fibrosis, which would involve the replacement of the cystic fibrosis transmembrane receptor gene. The vectors embodied in the present  
25 invention could also be used for treatment of hyperproliferative disorders such as rheumatoid arthritis or restenosis by transfer of genes encoding angiogenesis inhibitors or cell cycle inhibitors. Transfer of prodrug activators such as the HSV-TK gene can be also be used in the treatment of hyperploiferative disorders, including cancer.

## 2. Antisense constructs

Oncogenes such as *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl* and *abl* also are suitable targets. However, for therapeutic benefit, these oncogenes would be expressed as an antisense nucleic acid, so as to inhibit the expression of the oncogene.

5 The term "antisense nucleic acid" is intended to refer to the oligonucleotides complementary to the base sequences of oncogene-encoding DNA and RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target nucleic acid and interfere with transcription, RNA processing, transport and/or translation. Targeting double-stranded (ds) DNA with oligonucleotide leads to triple-helix formation; targeting RNA will lead to double-helix formation.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject. Nucleic acid sequences comprising "complementary nucleotides" are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, that the larger purines will base pair with the smaller pyrimidines to form only combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T), in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA.

As used herein, the terms "complementary" or "antisense sequences" mean nucleic acid sequences that are substantially complementary over their entire length and have very few base mismatches. For example, nucleic acid sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions with only single or double mismatches. Naturally, nucleic acid sequences which are "completely complementary" will be nucleic acid sequences which are entirely complementary throughout their entire length and have no base mismatches.



While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the constructs *in vitro* to determine whether the endogenous gene's function is affected or whether the expression of related genes having complementary sequences is affected.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in oncogene DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligo-nucleotides incorporating ribozyme sequences, or introduced into the cell as an expression construct encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense nucleic acids.

### 3. Antigens for Vaccines

Other therapeutics genes might include genes encoding antigens such as viral antigens, bacterial antigens, fungal antigens or parasitic antigens. Viruses include

picornavirus, coronavirus, togavirus, flavivirus, rhabdovirus, paramyxovirus, orthomyxovirus, bunyavirus, arenavirus, reovirus, retrovirus, papovavirus, parvovirus, herpesvirus, poxvirus, hepadnavirus, and spongiform virus. Preferred viral targets include influenza, herpes simplex virus 1 and 2, measles, small pox, polio or HIV.

- 5 Pathogens include trypanosomes, tapeworms, roundworms, helminths, . Also, tumor markers, such as fetal antigen or prostate specific antigen, may be targeted in this manner. Preferred examples include HIV env proteins and hepatitis B surface antigen. Administration of a vector according to the present invention for vaccination purposes would require that the vector-associated antigens be sufficiently non-immunogenic to
- 10 enable long term expression of the transgene, for which a strong immune response would be desired. Preferably, vaccination of an individual would only be required infrequently, such as yearly or biennially, and provide long term immunologic protection against the infectious agent.

#### 15 4. Control Regions

- In order for the viral vector to effect expression of a transcript encoding a therapeutic gene, the polynucleotide encoding the therapeutic gene will be under the transcriptional control of a promoter and a polyadenylation signal. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the host cell, or introduced
- 20 synthetic machinery, that is required to initiate the specific transcription of a gene. A polyadenylation signal refers to a DNA sequence recognized by the synthetic machinery of the host cell, or introduced synthetic machinery, that is required to direct the addition of a series of nucleotides on the end of the mRNA transcript for proper processing and trafficking of the transcript out of the nucleus into the cytoplasm for translation. The
- 25 phrase "under transcriptional control" means that the promoter is in the correct location in relation to the polynucleotide to control RNA polymerase initiation and expression of the polynucleotide.

- The term promoter will be used here to refer to a group of transcriptional control
- 30 modules that are clustered around the initiation site for RNA polymerase II. Much of the

thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately  
5 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters  
10 lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation.  
15 Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can  
20 be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

#### **E. Pharmaceutical Compositions**

25 In certain embodiments, the present invention also concerns formulations of a viral composition for administration to a mammal. It will also be understood that, if desired, the viral compositions disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, various pharmaceutically-active agents. As long as the compositions do not cause a significant adverse effect upon contact with the target cells

or host tissues, there is virtually no limit to other components which may also be included.

The formulation of pharmaceutically-acceptable excipients and carrier solutions are well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

### 1. Injectable Compositions and Delivery

The pharmaceutical compositions disclosed herein may be administered parenterally, intravenously, intramuscularly, or even intraperitoneally as described in U.S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated  
5 herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol,  
10 and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol,  
15 phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

20 For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill  
25 in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person  
30 responsible for administration will, in any event, determine the appropriate dose for the

individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

5 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those  
10 enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

15 The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be  
20 derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as  
25 injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such  
30 media and agents for pharmaceutical active substances is well known in the art. Except

insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

5           The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable  
10   for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

## 2. Oral Compositions and Delivery

Alternatively, the pharmaceutical compositions disclosed herein may be delivered  
15   via oral administration to an animal, and as such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

20   The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz *et al.*, 1997; Hwang *et al.*, 1998; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain  
25   the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may  
30   contain, in addition to materials of the above type, a liquid carrier. Various other

materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations:

For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. For example, a mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as those containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, including: gels, pastes, powders and slurries, or added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants, or alternatively fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

### 3. Nasal Delivery

The administration of agonist pharmaceutical compositions by intranasal sprays, inhalation, and/or other aerosol delivery vehicles is also considered. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs via nasal aerosol sprays has been described *e.g.*, in U. S. Patent 5,756,353 and U. S. Patent 5,804,212 (each specifically incorporated herein by reference in its entirety), and delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, 1998) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts.



Likewise, transmucosal drug delivery in the form of a polytetrafluoroethylene support matrix is described in U. S. Patent 5,780,045 (specifically incorporated herein by reference in its entirety).

5     **F.     EXAMPLES**

          The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute  
10    preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

15

**Example 1**  
                          **Materials and Methods**

**Lyophilizer**

          A Dura-stop  $\mu$ p lyophilizer (FTSsystems) with in process sample retrieving device  
20    was used. The lyophilizer is equipped with both thermocouple vacuum gauge and capacitance manometer for vacuum measurement. Condenser temperature is programmed to reach to -80°C. Vials were stoppered at the end of each run with a build-in mechanical stoppering device.

25    **Residual Moisture Measurement**

          Residual moisture in freeze dried product was analyzed by a Karl-Fisher type coulometer (Mettler DL37, KF coulometer).

**HPLC Analysis**

30    HPLC analysis of samples was done on a Beckman Gold HPLC system.

**Vials and Stoppers**

Borosilicate 3ml with 13mm opening lyo vials and their corresponding butyl rubber stoppers (both from Wheaton) were used for both lyophilization and liquid formulation development. The stoppered vials were capped with Flip-off aluminum caps using a capping device (LW312 Westcapper, The West Company).

5

## Example 2

### Lyophilization: Initial Cycle and Formulation Development

There are three main process variables that can be programmed to achieve optimal freeze-drying. Those are shelf temperature, chamber pressure, and lyophilization step duration time. To avoid cake collapse, shelf temperature need to be set at temperatures 2-3°C below the glass transition or eutectic temperature of the frozen formulation. Both the glass transition and eutectic temperatures of a formulation can be determined by differential scanning calorimetry (DSC) analysis. Chamber pressure is generally set at below the ice vapor pressure of the frozen formulation. The ice vapor pressure is dependent on the shelf temperature and chamber pressure. Too high a chamber pressure will reduce the drying rate by reducing the pressure differential between the ice and the surrounding, while too low a pressure will also slow down drying rate by reducing the heat transfer rate from the shelf to the vials. The development of a lyophilization cycle is closely related with the formulation and the vials chosen for lyophilization. The goal at this stage was to develop a somewhat conservative cycle to be able to successfully freeze dry a number of different formulations. The developed cycles and formulations will be further optimized when viruses are formulated in the formulations. Formulation excipient selection was based on the classical excipients found in most lyophilized pharmaceuticals. The excipients in a lyophilization formulation should provide the functions of bulking, cryoprotection, and lyoprotection. The excipients chosen were mannitol (bulking agent), sucrose (cryo- and lyoprotectant), and human serum albumin (HSA, lyoprotectant). These excipients were formulated in 10 mM Tris + 1 mM MgCl<sub>2</sub>, pH=7.50 at various percentages and filled into the 3 ml vials at a fill volume of 1 ml. To start with, a preliminary cycle was programmed to screen a variety of formulations based

on the criteria of residual moisture and physical appearance after drying. The cycle used is plotted in FIG. 1. Extensive screening was carried out by variation of the percentages of the individual excipients. Table 4 shows briefly some of the results.

5

**Table 4**  
**Evaluation of Different Formulations Under the Same Cycle**

<b>Formulation</b> <b>M%/S%/HSA %</b>	<b>Appearance</b>	<b>Moisture (% weight)</b>
10/5/0.5	good cake	0.89
5/5/0.5	good cake	1.5
3/5/0.5	loose cake (partial collapse)	3.4
1/5/0.5	no cake (collapse)	6.4

The results suggest that a minimum amount of 3% mannitol is required in the formulation in order to achieve pharmaceutically elegant cake. The percentages of sucrose in the formulation were also examined. No significant effect on freeze-drying was observed at sucrose concentrations of  $\leq 10\%$ . HSA concentration was kept constant to 0.5% during the initial screening stage.

After the evaluation of the formulations, freeze-drying cycle was optimized by changing the shelf temperature, chamber vacuum and the duration of each cycle step. Based on the extensive cycle optimization, the following cycle (cycle #14) was used for further virus lyophilization development.

1. Load sample at room temperature onto shelf.
2. Set shelf temperature to  $-45^{\circ}\text{C}$  and freeze sample. Step time 2 h.
3. Set shelf temperature at  $-45^{\circ}\text{C}$ , turn vacuum pump and set vacuum at 400mT. Step time 5 h.
4. Set shelf temperature at  $-35^{\circ}\text{C}$ , set vacuum at 200mT. Step time 13 h.
5. Set shelf temperature at  $-22^{\circ}\text{C}$ , set vacuum at 100mT. Step time 15 h.
6. Set shelf temperature at  $-10^{\circ}\text{C}$ , set vacuum at 100mT. Step time 5 h.
7. Set shelf temperature at  $10^{\circ}\text{C}$ , set vacuum at 100mT. Step time 4 h.
8. Vial stoppering under vacuum.

### Example 3

#### Cycle and Formulation Development With Virus in Formulation

Effect of Sucrose Concentration in Formulation. Cycle and formulation were further optimized according to virus recovery after lyophilization analyzed by both HPLC and plaque forming unit (PFU) assays. Table 5 shows the virus recoveries immediate after drying in different formulations using the above drying cycle. Variation of the percentage of sucrose in the formulation had significant effect on virus recoveries.

10

**Table 5**  
**Recoveries of Virus After Lyophilization**

Formulation M%/S%/HSA %	Appearance	Residual moisture	Recovery (%)
6/0/0.5	good cake	0.44%	0
6/3.5/0.5	good cake	2.2%	56
6/5/0.5	good cake	2.5%	81
6/6/0.5	good cake	2.7%	120
6/7/0.5	good cake	2.8%	120
6/8/0.5	good cake	3.3%	93
6/9/0.5	good cake	3.7%	120

Residual moisture in the freeze-dried product increased as the sucrose percentage increased. A minimum sucrose concentration of 5% is required in the formulation to maintain a good virus recovery after lyophilization. Similar sucrose effects in formulation that had 5% instead of 6% mannitol were observed. However, good virus recovery immediately after drying does not necessary support a good long-term storage stability. As a result, formulations having 4 different sucrose concentrations of 6, 7, 8, and 9%, were incorporated for further evaluation.

20

Effect of HSA in Formulation. The contribution of HSA concentrations in the formulation on virus recovery after drying was examined using the same freeze drying cycle. Table 6 shows the results.

**Table 6**  
**Effects of HSA Concentration on Lyophilization**

Formulation M%/S%/o/HSA %	Appearance	Residual moisture	Recovery (%)
6/7/0	Good cake	0.98	83
6/7/0.5	Good cake	1.24	120
6/7/2	Good cake	1.5	110
6/7/5	Good cake	1.7	102

The results indicate that inclusion of HSA in the formulation had positive effect on virus recovery after drying. Concentrations higher than 0.5% did not further improve the virus recovery post drying. As a result, 0.5% HSA is formulated in all the lyophilization formulations.

Cycle Optimization. As indicated in Table 5, relatively high residual moistures were present in the dried product. Although there has not been a known optimal residual moisture for freeze dried viruses, it could be beneficial for long term storage stability to further reduce the residual moisture in the dried product. After reviewing of the drying cycle, it was decided to increase the secondary drying temperature from 10°C to 30°C without increasing the total cycle time. As indicated in Table 7, significant reduction in residual moisture had been achieved in all the formulations without negative effects on virus recoveries. With the improved drying cycle, residual moisture was less than 2% in all the formulations immediately after drying. It is expected that the reduced residual moisture will improve the long-term storage stability of the dried product.

**Table 7**  
**Effects of Secondary Drying Temperature on Lyophilization**

Formulation M%/S%/HSA %	Secondary drying at 10°C		Secondary drying at 30°C	
	Residual moisture (w %)	Recovery (%)	Residual moisture	Recovery
6/6/0.5	2.2	100	0.8	93
6/7/0.5	2.5	86	1.1	100
6/8/0.5	2.7	83	1.3	87
6/9/0.5	3.3	93	1.5	86
5/6/0.5	2.3	110	1.0	94

5/7/0.5	2.7	88	1.2	85
5/8/0.5	3.5	97	1.6	88
5/9/0.5	4	90	1.9	86

N<sub>2</sub> Backfilling (Blanketing). Lyophilization was done similarly as above except that dry N<sub>2</sub> was used for gas bleeding for pressure control during the drying and backfilling at the end of the cycle. At the end of a drying run, the chamber was filled with dry N<sub>2</sub> to about 80% atmospheric pressure. Subsequently, the vials were stoppered. No difference was noticed between the air and N<sub>2</sub> blanketing runs immediate after drying. However, if oxygen present in the vial during air backfilling causes damaging effect (oxidation) on the virus or excipients used during long-term storage, backfilling with dry N<sub>2</sub> is likely to ameliorate the damaging effects and improve long term storage stability of the virus.

Removal of Glycerol From Formulation. During the preparation of virus containing formulations, stock virus solution was added to the pre-formulated formulations at a dilution factor of 10. Because of the presence of 10% glycerol in the stock virus solution, 1% glycerol was introduced into the formulations. To examine any possible effect of the presence of 1% glycerol on lyophilization, a freeze drying run was conducted using virus diafiltered into the formulation of 5% (M)/7%(S)/0.5% (HSA). Diafiltration was done with 5 vol. of buffer exchange using a constant volume buffer exchange mode to ensure adequate removal of residual glycerol (99% removal). After diafiltration, virus solution was filled into vials and then lyophilized similarly. Table 8 shows the lyophilization results.

**Table 8**  
**Lyophilization without Glycerol**

Formulation M%/S%/HSA %	Residual moisture	Recovery (%)
5/7/0.5	1.0	80

No significant difference after freeze drying was observed between formulations with and without 1% glycerol. Possible implications of this change on long term storage will be evaluated.

5

#### Example 4

#### Long Term Storage Stability Study

Adp53 virus lyophilized under different formulations and different cycles was placed at -20°C, 4°C, and room temperature (RT) under dark for long term storage stability evaluation. Parameters measured during the stability study were PFU, HPLC viral particles, residual moisture, and vacuum inside vial. The plan is to be able to evaluate virus stability at various conditions for up to one-year storage. Table 6 shows the data after 12-month storage with secondary drying at 10°C without N<sub>2</sub> blanketing. Lyophilized virus is stable at both -20°C and 4°C storage for up to 12 months. However, virus was not stable at room temperature storage. More than 50% loss in infectivity was observed at RT after 1-month storage. The reason for the quick loss of infectivity at RT is not clear. However, it is likely that RT is above the glass transition temperature of the dried formulation and resulting in the accelerated virus degradation. A differential scanning calorimetry (DSC) analysis of the formulation could provide very useful information. Pressure change inside the vials during storage was not detected, which indicates that the vials maintained their integrity. The slight increase in residual moisture during storage can be attributed to the release of moisture from the rubber stopper into the dried product.

Table 9  
Secondary Drying at 10°C  
Formulation Set 10 (6-9)

Date*	PFU×10 <sup>9</sup> /ml				HPLC Viral Particle (×10 <sup>10</sup> /ml)				Water Content (W%)			
	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9
4/11/97	5.5	6.0	5.8	6.5	24.5	24.6	24.9	26.7	2.2	2.5	2.7	3.3
5/15/97 <sup>a</sup>	7.6	7.1	7.5	8.1	22.4	25.6	26.8	28.5	2.2	2.5	2.8	3.3
5/15/97 <sup>b</sup>	6.6	6.3	6.5	10.0	22.0	23.0	24.0	27.5	2.4	2.6	3.0	3.4
5/15/96 <sup>c</sup>	7.1	7.1	6.7	3.3	14.5	16.5	6.2	4.2	2.7	2.9	3.2	3.5
7/18/97 <sup>a</sup>	6.8	6.4	6.8	7.2	28.7	28.9	28.6	31.2	2.3	2.5	2.8	3.3
7/18/97 <sup>b</sup>	6.0	5.8	7.3	9.0	25.0	26.6	27.6	31.1	2.5	2.8	3.0	3.6
7/18/97 <sup>c</sup>	1.2	0.8	4.0	1.4	0.9	1.8	0.7	0.7	2.7	2.9	3.0	3.4
10/22/97 <sup>a</sup>	7.9	7.5	7.9	7.8	25.5	25.0	25.4	26.2	2.4	2.6	2.8	3.1
10/22/97 <sup>b</sup>	6.8	6.8	5.8	8.0	22.0	23.0	24.7	24.2	2.7	2.9	3.2	3.6
10/22/97 <sup>c</sup>	<0.01	<0.01	<0.01	<0.01	N.D.	N.D.	N.D.	N.D.	2.7	2.9	3.1	3.4
4/16/98 <sup>a</sup>	6.0	5.8	7.1	7.2	19.3	20.3	23.5	26.1	2.4	2.6	3.0	3.4
4/16/98 <sup>b</sup>	5.4	7.2	6.1	6.3	21.7	22.8	22.9	24.6	2.9	3.1	3.3	3.8
4/16/98 <sup>c</sup>	0.0003	0.001	0.0007	0.001	N.D.	N.D.	N.D.	N.D.	2.7	2.9	3.1	3.4

\*Temp. <sup>a</sup>(-20°C) <sup>b</sup>(4°C) <sup>c</sup>(r.t.)



## Controls

Date	PFU $\times 10^9$ /ml				HPLC Viral Particle ( $\times 10^{10}$ /ml)			
	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9
4/11/97	5.5	7.0	7.0	7.0	35.5	35.8	36.0	36.9

N.D.: not detectable

Formulation set 10: 6%-mannitol, 0.5% HSA, 1% glycerol and different percentages of sucrose in 10 mM-tris buffer pH = 7.5, 1 mM MgCl<sub>2</sub>

Table 9 Continued  
Formulation Set 11 (6-9)

Date*	PFU $\times 10^9$ /ml			HPLC Viral Particle ( $\times 10^{10}$ /ml)						Water Content (W%)		
	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9
5/2/97	7.0	6.0	6.3	5.8	28.5	28.8	28.4	29.5	2.3	2.7	3.5	4.0
6/20/97 <sup>a</sup>	6.2	6.6	6.9	6.5	26.4	25.0	27.0	27.3	2.2	2.8	3.4	4.6
6/20/97 <sup>b</sup>	6.1	6.0	6.5	6.5	24.1	22.1	25.6	26.6	2.5	2.8	3.5	4.8
6/20/97 <sup>c</sup>	3.3	3.0	1.0	<0.1	20.5	17.4	5.2	9.1	2.7	3.1	3.5	4.7
8/18/97 <sup>a</sup>	8.0	7.2	7.5	7.6	21.6	21.8	25.3	24.9	2.3	2.8	3.7	4.9
8/18/97 <sup>b</sup>	8.0	7.3	8.0	8.0	22.7	22.7	24.9	25.0	2.6	3	3.9	4.2
8/18/97 <sup>c</sup>	<0.1	<0.1	<0.1	<0.1	N.D.	N.D.	0.2	13.1	2.7	3.0	3.5	4.4
10/22/97 <sup>a</sup>	7.9	7.5	7.9	6.7	21.0	22.0	25.1	24.0	2.4	3.0	3.9	4.4
10/22/97 <sup>b</sup>	6.0	6.9	6.8	7.3	21.4	22.0	23.1	23.1	2.6	3.0	3.3	4.6
10/22/97 <sup>c</sup>	<0.01	<0.01	<0.01	<0.015	N.D.	N.D.	N.D.	9.0	2.7	2.9	3.9	5.0
5/8/98 <sup>a</sup>	8.3	7.5	8.0	8.7	19.0	18.2	19.9	21.1	2.6	3.1	4.0	4.6
5/8/98 <sup>b</sup>	7.0	7.1	7.8	6.5	17.3	17.1	18.2	17.8	2.8	3.2	4.1	5.1
5/8/98 <sup>c</sup>	0.00033	0.000065	0.00045	0.000016	N.D.	N.D.	N.D.	N.D.	2.7	2.9	4.0	4.9

\*Temp. <sup>a</sup>(-20°C) <sup>b</sup>(4°C) <sup>c</sup>(R.T.)

## Controls

Date	PFU $\times 10^9$ /ml		HPLC Viral Particle ( $\times 10^{10}$ /ml)					
	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9
5/2/97	6.4	6.8	6.5	6.5	37.7	35.7	37.3	36.0

N.D.: not detectable

Formulation set 11: 5%-mannitol, 0.5% HSA, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH = 7.5, 1 mM MgCl<sub>2</sub>)  
F11-(6-9)R1-S

**Table 10**  
**Secondary Drying at 30°C Without N<sub>2</sub> Blanketing**  
**Formulation Set 10 (6-9)**

Date*	PFU×10 <sup>9</sup> /ml			HPLC Viral Particle (×10 <sup>10</sup> /ml)						Water Content (W%)			
	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-10	Set 10-6	Set 10-7	Set 10-8	Set 10-9
5/15/97	6.5	5.6	6.1	6.0	18.0	18.6	21.9	23.3	0.8	1.1	1.3	1.5	1.5
6/20/97 <sup>b</sup>	5.4	5.6	5.5	5.5	14.6	14.9	17.2	16.6	0.8	1.2	1.5	1.6	1.6
6/20/97 <sup>c</sup>	4.5	5.0	5.5	6.0	10.8	11.8	15.0	15.4	1.3	1.4	1.6	1.9	1.9
8/18/97 <sup>b</sup>	7.0	6.7	6.8	7.0	15.3	17.1	17.9	17.7	1.3	1.5	1.5	1.7	1.7
8/18/97 <sup>c</sup>	2.4	2.2	4.8	5.8	4.3	7.2	11.7	14.2	1.3	1.6	1.7	2.1	2.1
11/20/97 <sup>b</sup>	5.5	5.5	5.3	5.7	21.9	21.9	27.2	26.4	1.1	1.4	1.6	1.9	1.9
11/20/97 <sup>c</sup>	0.5	0.9	2.3	3.1	1.5	6.3	8.8	13.5	1.3	1.7	1.8	2.2	2.2
5/14/98 <sup>ab</sup>	4.9	4.7	5.4	6.5	9.7	11.9	12.6	14.2	1.2	1.6	2.2	1.4	1.4
5/14/98 <sup>c</sup>	0.000006	0.00006	0.00004	0.000024	N.D.	N.D.	N.D.	N.D.	1.4	1.6	1.3	2.0	2.0

\*Temp. <sup>ab</sup>(4°C)      °(R.T.)

Controls									
Date	PFU $\times 10^9$ /ml			HPLC Viral Particle ( $\times 10^{10}$ /ml)					
	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9	
5/15/97	7.0	5.6	7.0	7.0	31.2	30.6	31.6	31.4	

Formulation set 10: 6%-mannitol, 0.5% HSA, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH = 7.5, 1 mM MgCl<sub>2</sub>)

F10(6-9)R2-S

Table 10 - Continued  
Formulation Set 11 (6-9)

Date*	PFU×10 <sup>9</sup> /ml				HPLC Viral Particle (×10 <sup>10</sup> /ml)				Water Content (W%)			
	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9
5/22/97	7.5	6.3	7.3	6.5	17.4	16.6	20.3	24.7	1.0	1.2	1.6	1.9
6/20/97 <sup>b</sup>	5.5	6.3	6.0	7.5	14.8	16.1	17.5	21.1	1.2	1.3	1.7	1.8
6/20/97 <sup>c</sup>	5.0	6.0	6.0	7.5	12.6	14.9	17.2	20.3	1.4	1.6	1.9	2.0
8/18/97 <sup>b</sup>	6.3	6.7	68	7.5	15.7	17.2	18.5	22.6	1.2	1.5	1.8	1.9
8/18/97 <sup>c</sup>	3.3	4.5	5.5	7.0	7.4	10.5	15.8	21.2	1.6	1.7	1.9	2.2
11/20/97 <sup>b</sup>	5.3	5.6	5.3	6.6	22.6	26.4	30.0	35.0	1.2	1.4	1.9	1.9
11/20/97 <sup>c</sup>	0.8	1.9	3.0	0.1	3.2	9.6	18.3	1.3	1.6	1.7	2.0	2.1
5/14/98 <sup>b</sup>	6.7	7.2	6.9	7.6	12.4	13.9	15.5	18.5	1.3	1.6	2.0	2.2
5/14/98 <sup>c</sup>	0.0013	0.00005	0.00031	0.00045	N.D.	N.D.	N.D.	N.D.	1.6	1.8	1.6	2.0

\*Temp. <sup>a</sup>(-20°C) <sup>b</sup>(4°C) <sup>c</sup>(R.T.)

Controls									
Date	PFU×10 <sup>9</sup> /ml			HPLC Viral Particle (×10 <sup>10</sup> /ml)					
	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9	
5/22/97	8.0	7.4	8.3	7.6	26.7	27.6	27.5	32.4	

Formulation set 11: 5%-mannitol, 0.5% HSA, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH = 7.5, 1 mM MgCl<sub>2</sub>)

F11-(6-9)R2-S

Table 11  
Secondary Drying at 30°C With N<sub>2</sub> Blanketing  
Formulation Set 10 (6-9) + Adp53

Date*	PFU×10 <sup>9</sup> /ml				HPLC Viral Particle (×10 <sup>10</sup> /ml)				Water Content (W%)			
	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9
6/13/97	3.4	4.3	4.1	4.2	16.0	16.5	16.1	18.1	0.8	1.1	1.3	1.4
7/18/97 <sup>b</sup>	6.3	6.3	6.0	6.0	17.9	19.5	19.9	20.6	0.9	1.2	1.4	1.6
7/18/97 <sup>c</sup>	4.1	5.5	5.0	5.5	11.4	15.5	18.2	20.6	1.2	1.4	1.7	1.8
9/16/97 <sup>b</sup>	4.2	5.5	4.5	5.1	15.3	16.1	16.4	17.8	1.0	1.3	1.5	1.7
9/16/97 <sup>c</sup>	0.7	1.2	5.0	4.0	2.9	5.0	9.5	13.0	1.3	1.5	1.8	2.0
12/4/97 <sup>b</sup>	5.5	5.3	5.4	5.9	16.1	16.2	18.1	18.5	1.1	1.4	1.6	1.7
12/4/97 <sup>c</sup>	0.3	0.5	2.5	3.4	N.D.	1.7	4.7	8.8	1.4	1.6	1.8	2.0
6/29/98 <sup>ab</sup>	3.8	5.1	5.3	5.4	10.6	10.8	12.0	12.9	1.3	1.5	1.8	1.9
6/29/98 <sup>c</sup>	0.00003	0.00006	0.0001	0.0001	N.D.	N.D.	N.D.	N.D.	1.4	1.6	1.7	1.8

\*Temp. <sup>ab</sup>(4°C)      °(R.T.)



## Controls

Date	PFU $\times 10^9$ /ml				HPLC Viral Particle ( $\times 10^{10}$ /ml)			
	Set 10-6	Set 10-7	Set 10-8	Set 10-9	Set 10-6	Set 10-7	Set 10-8	Set 10-9
6/13/97	4.7	3.8	5.5	6.2	26.0	26.2	27.4	27.5

Formulation set 10: 6%-mannitol, 0.5% HSA, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH: 7.5, 1 mM MgCl<sub>2</sub>)

F11-(6-9)R3-S

Table 11  
Continued Formulation set 11 (6-9) + Adp53

Date*	PFU×10 <sup>9</sup> /ml				HPLC Viral Particle (×10 <sup>10</sup> /ml)				Water Content (W%)			
	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7	Set 11-8	Set 11-9
6/13/97	3.4	4.2	3.6	4.4	16.1	16.3	18.4	19.3	0.9	1.3	1.8	1.9
7/18/97 <sup>b</sup>	5.5	6.2	6.5	6.2	18.0	19.5	23.0	23.9	1.0	1.4	1.8	2.1
7/18/97 <sup>c</sup>	3.7	6.0	6.7	7.3	13.7	18.7	21.8	22.8	1.3	1.7	2.0	2.2
9/16/97 <sup>b</sup>	3.9	4	4.6	6	15.6	17.3	19.5	20.6	1.3	1.5	1.9	2.1
9/16/97 <sup>c</sup>	0.78	2.2	4.0	5.3	3.6	6.8	13.8	14.6	1.5	1.9	2.3	2.4
12/4/97 <sup>b</sup>	4.6	5.3	8.0	6.1	15.7	18.2	21.4	21.6	1.2	1.6	2.1	2.2
12/4/97 <sup>c</sup>	0.4	0.6	0.3	0.01	N.D.	N.D.	1.7	N.D.	1.6	1.8	2.1	2.1
6/29/98 <sup>ab</sup>	4.9	5.0	5.4	6.4	11.4	14.2	13.7	16.0	1.5	1.7	2.1	2.6
6/29/98 <sup>c</sup>	0.0001	0.00015	0.00085	0.0012	N.D.	N.D.	N.D.	N.D.	1.6	1.7	2.2	2.3

\*Temp. <sup>ab</sup>(4°C)      °(R.T.)

## Controls

Date	PFU $\times 10^9$ /ml			HPLC Viral Particle ( $\times 10^{10}$ /ml)		
	Set 11-6	Set 11-7	Set 11-8	Set 11-9	Set 11-6	Set 11-7
6/13/97	4.5	5.0	4.0	5.0	26.5	26.9
					26.6	27.1

Formulation set 11: 6%-mannitol, 0.5% HSA, 1%-glycerol and different percentages of sucrose in 10 mM-tris buffer (pH = 7.5, 1 mM MgCl<sub>2</sub>)  
F11-(6-9)R3-S

Table 12  
Aqueous Formulation Set #1

Date (Storage Conds.)	PFU x10 <sup>9</sup> /ml			
	10%-G	5%-S+5%-HSA	5%-S+1%-PEG	5%-T+1%-PEG
8/1/97	5.8	4.7	4.3	4.4
8/28/97 (4°C, N <sub>2</sub> )	5.8	5.8	6.4	6.3
8/28/97 (4°C, Air)	5.0	5.9	6.0	5.9
8/28/97 (R.T., N <sub>2</sub> )	4.4	4.8	5.0	6.0
8/28/97 (R.T., Air)	4.3	5.0	5.0	5.6
10/30/97 (4°C, N <sub>2</sub> )	3.8	4.0	4.7	3.8
10/30/97 (4°C, Air)	3.0	4.1	3.7	4.7
10/30/97 (R.T., N <sub>2</sub> )	1.5	3.4	3.5	3.6
10/30/97 (R.T., Air)	1.5	3.6	2.2	3.1
1/12/98 (4°C, N <sub>2</sub> )	3.2	4.1	3.3	3.4
1/12/98 (4°C, Air)	1.5	3.8	3.9	3.4
1/12/98 (R.T., N <sub>2</sub> )	0.1	1.4	0.7	0.7
1/12/98 (R.T., Air)	0.4	1.6	1.0	0.4
4/30/98 (4°C, N <sub>2</sub> )	0.08	4.3	4.0	5.3
4/30/98 (4°C, Air)	1.5	3.6	4.4	4.5
4/30/98 (R.T., N <sub>2</sub> )	0.0025	0.23	0.11	0.17
4/30/98 (R.T., Air)	0.0015	0.21	0.063	0.007
2/5/99 (4°C, N <sub>2</sub> )	0.0005	5.8	4.1	3.9
2/5/99 (4°C, Air)	0.02	4.7	4.3	4.5
2/5/99 (R.T., N <sub>2</sub> )	<10 <sup>2</sup>	0.0007	<10 <sup>4</sup>	0.0002
2/5/99 (R.T., Air)	2x10 <sup>2</sup>	0.0002	0.0003	2x10 <sup>3</sup>

Date (Storage Conds.)	HPLC Viral Particle ( $\times 10^{10}/\text{ml}$ )				
	10%-G	5%-S+5%-HSA	5%-S+1%-PEG	5%-T+1%-PEG	
8/1/97	16.9	14.5	16.1	16.7	
8/28/97 (4°C, N <sub>2</sub> )	13.3	14.9	13.8	13.4	
8/28/97 (4°C, Air)	12.9	14.2	12.9	12.9	
8/28/97 (R.T., N <sub>2</sub> )	12.6	14.5	13.5	12.9	
8/28/97 (R.T., Air)	12.3	13.7	13.0	13.0	
10/30/97 (4°C, N <sub>2</sub> )	14.0	15.5	14.7	14.8	
10/30/97 (4°C, Air)	12.6	14.9	14.3	14.4	
10/30/97 (R.T., N <sub>2</sub> )	13.8	15.1	14.6	14.4	
10/30/97 (R.T., Air)	12.7	14.7	14.8	14.4	
1/12/98 (4°C, N <sub>2</sub> )	7.3	11.1	9.5	9.5	
1/12/98 (4°C, Air)	7.7	10.8	10.2	10.0	
1/12/98 (R.T., N <sub>2</sub> )	10.0	10.8	11.1	10.4	
1/12/98 (R.T., Air)	9.9	11.0	10.0	10.4	
4/30/98 (4°C, N <sub>2</sub> )	5.1	12.3	12.3	12.1	
4/30/98 (4°C, Air)	5.0	11.6	11.8	11.9	
4/30/98 (R.T., N <sub>2</sub> )	11.1	12.3	12.6	12.5	
4/30/98 (R.T., Air)	11.0	12.4	12.3	11.0	
2/5/99 (4°C, N <sub>2</sub> )	3.4	5.8	11.4	11.0	
2/5/99 (4°C, Air)	3.9	7.1	11.0	11.2	
2/5/99 (R.T., N <sub>2</sub> )	10.1	7.9	8.5	10.9	
2/5/99 (R.T., Air)	9.7	7.1	10.3	9.3	

G: glycerol S: sucrose PEG: PEG-3500 T: trehalose

Glycerol: 10% glycerol in DPBS buffer

Other formulations are in 10 mM-tris buffer with 0.15 M-NaCl and 1 mM-MgCl<sub>2</sub> (pH = 8.2).

Table 13

## Aqueous Formulation Set #2

Date (Temp.)	PFU $\times 10^9$ /ml						
	AQF2-1	AQF2-2	AQF2-3	AQF2-4	AQF2-5	AQF2-6	AQF2-7*
9/25/97	2.8	2.8	2.8	3.0	2.8	2.8	2.7
11/05/97 (4 °C)	2.3	3.2	2.4	3.6	2.7	2.0	3.6
11/05/97 (R.T.)	1.4	1.9	1.3	1.5	2.4	2.5	3.1
12/12/97 (4°C)	2.2	0.1	2.4	2.7	2.1	2.1	3.2
1/09/98 (R.T.)	1.2	0.1	0.2	1.2	0.2	0.1	1.3

Date (Temp.)	PFU $\times 10^9$ /ml			
	AQF2-8*	AQF2-9*	AQF2-10*	AQF2-11* AQF2-12
9/25/97	2.8	2.7	3.3	3.1 2.7
11/05/97 (4 °C)	3.8	2.7	3.0	3.5 2.5
11/05/97 (R.T.)	3.3	3.1	4.1	2.8 1.1
12/12/97 (4°C)	2.1	3.0	3.0	3.4 2.9
1/09/98 (R.T.)	1.1	0.2	0.1	20 1.1

\* Gave better recovery.

Date (Temp.)	HPLC viral particle ( $\times 10^{10}$ /ml)					
	AQF2-1	AQF2-2	AQF2-3	AQF2-4	AQF2-5	AQF2-6 AQF2-7
9/25/97	10.9	9.6	9.7	11.3	10.7	10.6 10.9
11/05/97 (4 °C)	7.9	7.6	8.7	8.8	8.9	7.5 8.6
11/05/97 (R.T.)	8.2	6.6	7.6	8.6	7.7	9.3 9.0
12/12/97 (4°C)	6.7	1.5	8.0	6.9	5.2	7.5 7.5
12/17/97 (R.T.)	7.0	1.2	7.0	7.5	4.1	7.1 7.0

Date (Temp.)	HPLC viral particle $\times 10^{10}$ /ml		
	AQF2-8	AQF2-9	AQF2-10 AQF2-11 AQF2-12

9/25/97	10.8	10.7	11.4	11.8	10.7
11/05/97 (4 °C)	9.1	9.2	10.3	11.2	9.6
11/05/97 (R.T.)	8.0	9.3	10.3	11.1	9.6
12/12/97 (4°C)	6.1	7.6	8.8	7.3	7.7
12/17/97 (R.T.)	3.0	8.2	7.6	8.4	7.5

Aqueous Formulation Set 2

Excipients	AQF2-1	AQF2-2	AQF2-3	AQF2-4	AQF2-5	AQF2-6	AQF2-7
mannitol (W%)	5	5	5				5
sucrose (W%)				5	5	5	5
glycine (M)	0.25			0.25			0.25
arginine (M)		0.25			0.25		
urea (W%)			1			1	
peg (w%)							

Excipients	AQF2-8	AQF2-9	AQF2-10	AQF2-11	AQF2-12
mannitol (W%)	5	5	5	5	
sucrose (W%)	5	5	5	5	10
glycine (M)				0.25	0.25
arginine (M)	0.25			0.25	
urea (W%)		1		1	
peg (w%)			1	1	

Formulations are in 10 mM-tris buffer (pH = 7.5) which consists of 1% glycerol and 1 mM MgCl<sub>2</sub>.

The formulations are stored at 4 °C and room temperature under nitrogen.

Table 14  
Aqueous Formulation Set #3

Date (temp.)	PFU × 10 <sup>9</sup>					HPLC Viral Particle (×10 <sup>9</sup> /ml)				
	F10-7	F10-8	F11-7	F11-8	F10-7	F10-8	F11-7	F11-8		
10/3/97	2.2	3.3	2.1	2.8	12.1	12.0	11.8	12.0		
11/6/97(-20°C)	3.4	4.0	2.8	3.4	10.6	10.5	10.1	10.3		
11/6/97(4°C)	3.5	3.6	4.3	2.8	10.0	9.7	9.9	10.3		
1/15/98 (-20°C)	3.8	4.8	3.2	3.7	7.3	7.4	7.7	8.0		



1/15/98 (4°C)	3.5	3.1	2.9	3.1	7.5	7.4	7.6	7.5
Excipients	F10-7	F10-8	F11-7	F11-8				
mannitol(W%)	6	6	5	5				
sucrose(VV%)	7	8	7	8				
HSA(W%)	0.5	0.5	0.5	0.5				
glycerol(W%)	1	1	1	1				
MgCl <sub>2</sub> (mM)	1	1	1	1				

Table 15  
Liquid formulation set #4

Date (Temp.)	PFU ( $\times 10^9$ /ml)						
	AQF4-1	AQF4-2	AQF4-3	AQF4-4	AQF4-5	AQF4-6	AQF4-7
1/13/98	3.0	2.5	3.6	3.4	2.7	3.1	3.4
2/16/98 (4°C)	2.5	3.2	3.3	2.9	2.6	2.9	2.6
2/16/98 (R.T.)	1.8	2.7	1.6	3.6	2.6	1.6	1.7
4/10/98 (4°C)	2.2	2.0	2.6	3.0	2.4	1.9	2.2
4/10/98 (R.T.)	0.4	0.4	0.3	0.5	0.4	<0.1	1.1
7/24/98 (4°C)	2.4	2.8	2.6	3.5	1.9	2.2	2.6
7/24/98 (R.T.)	0.002	0.005	0.006	0.005	0.005	0.005	0.001
1/8/99 (4°C)	2.9	2.4	2.1	2.6	2.0	2.2	2.1
1/8/99 (R.T.)	0.0002	0.0004	0.0004	0.0002	0.0004	0.0004	0.00006

Date (Temp.)	HPLC Viral Particles ( $\times 10^{10}$ /ml)						
	AQF4-1	AQF4-2	AQF4-3	AQF4-4	AQF4-5	AQF4-6	AQF4-7
1/13/98	7.2	8.8	9.2	9.0	7.8	7.9	9.1
2/16/98 (4°C)	7.5	9.3	9.2	9.5	8.2	8.4	9.6
2/16/98 (R.T.)	6.8	9.0	9.5	9.0	8.7	8.4	9.3
4/10/98 (4°C)	7.1	9.2	9.6	9.6	8.9	9.1	9.9
4/10/98 (R.T.)	7.5	9.5	10.1	9.7	8.9	8.9	9.5
7/24/98 (4°C)	8.1	9.9	11.1	10.3	9.2	7.4	9.3
7/24/98 (R.T.)	7.3	3.0	10.7	8.9	10.4	10.45	3.5
1/8/99 (4°C)	7.8	10.3	10.3	10.1	8.7	1.7	9.5
1/8/99 (R.T.)	8.4	11.0	11.3	11.0	9.7	10.4	9.4
Excipients	AQF4-1	AQF4-2	AQF4-3	AQF4-4	AQF4-5	AQF4-6	AQF4-7

Mannitol (w%)	5	5	5	5	5	5	5
Sucrose (w%)	5	5	5	5	5	5	5
Tween -80 (w%)		0.02	0.1	0.5	0.02	0.1	0.5
Chap (w%)							

Buffer: 10 mM Tris+H<sub>2</sub>O.15M NaCl+1mMMgCl<sub>2</sub>, pH = 8.2  
Formulations were blanketed with N<sub>2</sub>.

Table 10 and Table 11 show the storage stability data with secondary drying at 30°C without and with N<sub>2</sub> backfilling, respectively. Because of the nearly identical stability observed at -20°C and 4°C storage conditions, and to reduce the consumption of virus, -20°C was not included in the long-term storage stability study. Similar to the samples dried with secondary drying at 10°C, virus is stable at 4°C but not stable at RT. However, relative better stability was observed at RT storage than those dried at 10°C secondary drying. This is likely to be the result of the lower residual moisture attained at 30°C secondary drying. This result suggests that residual moisture is an important parameter that affects storage stability during long term storage.

HPLC viral particle recoveries are consistently lower than virus recoveries calculated from PFU assay immediate after drying. The reason for the discrepancy is not clear. However, it is likely to be related to possible virus aggregation during freeze-drying. Electron microscopy evaluation is being carried out to examine possible virus aggregation after lyophilization. During storage, HPLC analysis indicates that virus is stable at both -20°C and 4°C storage and not stable at RT, which is consistent with the results from PFU assay.

### Example 5

#### HSA Alternatives

The presence of HSA in the formulations could be a potential regulatory concern. As a result, a variety of excipients have been evaluated to substitute HSA in the formulation. The substitutes examined included PEG, amino acids (glycine, arginine), polymers (polyvinylpyrrolidone), and surfactants (Tween-20 and Tween-80). These HSA substitutes are, however, suboptimal relative to HSA. Effort on further development was minimal.

### Example 6

#### Liquid Formulation

Concurrent with the development of lyophilization of Adp53 product, experimentation was carried out to examine the possibility of developing a liquid formulation for Adp53 product. The goal was to develop a formulation that can provide enough stability to the virus when stored at above freezing temperatures. Four sets of liquid formulations have been evaluated. In the first set of formulation, the current 10% glycerol formulation was compared to HSA and PEG containing formulations. In the second set of formulation, various amino acids were examined for formulating Adp53. In the third set of formulation, the optimal formulation developed for lyophilization was used to formulate Adp53 in a liquid form. In the fourth set of formulation, detergents were evaluated for formulating Adp53. Viruses formulated with all those different formulations are being tested for long term storage stability at -20°C, 4°C, and RT.

#### Liquid Formulation Set #1

HSA containing formulation (5% sucrose+ 5% HSA in 10 mM Tris buffer, 150 mM NaCl, and 1 mM MgCl<sub>2</sub>, pH=8.20 buffer) was compared with 10% glycerol in DPBS buffer and sucrose/PEG and Trehalose/PEG formulations. PEG has been recommended as a good preferential exclusion agent in formulations (Wong and Parasrampurita, 1997). It is included in this set of formulation to examine whether it can provide stabilization effect on Adp53. Formulations were filled into the 3 ml lyo vials at a fill volume of 0.5 ml. Vials were capped under either atmospheric or N<sub>2</sub> blanketing conditions to examine any positive effects N<sub>2</sub> blanketing may have on long term storage stability of Adp53. To ensure adequate degassing from the formulation and subsequent N<sub>2</sub> blanketing, the filled vials was partially stoppered with lyo stoppers and loaded onto the shelf of the lyophilizer under RT. The lyophilizer chamber was closed and vacuum was established by turning on the vacuum pump. The chamber was evacuated to 25 in Hg. Then the chamber was purged completely with dry N<sub>2</sub>. The evacuation and gassing were repeated twice to ensure complete N<sub>2</sub> blanketing. N<sub>2</sub> blanketed vials were placed

with the non-N<sub>2</sub> blanketed vials at various storage conditions for storage stability evaluation. Table 12 shows the analysis data for up to 18 months storage at 4°C and RT.

5 Statistically significant drops in virus PFU and HPLC viral particles were observed for 10% glycerol formulation after 3 months storage at both 4°C and RT. No statistically significant virus degradation was observed for all other formulations at 4°C storage. However, decrease in virus infectivity was observed when stored at RT.

#### **Liquid Formulation Set #2**

10 Various combinations of amino acids, sugars, PEG and urea were evaluated for Adp53 stabilization during long storage. Table 13 shows the 12-month stability data. The results indicate that combination of 5% mannitol and 5% sucrose with other excipients gave better storage stability at RT for one month. Adp53 is most stable in formulation has all the excipients. In this set of formulation, no human or animal derived  
15 excipients were included. It is our expectation to develop a liquid formulation without including any proteins derived from either human or animal origins.

#### **Liquid Formulation Set #3**

20 The optimal formulations developed for lyophilization was evaluated for formulating Adp53 in a liquid form. This approach would be a good bridging between liquid formulation and lyophilization if satisfactory Adp53 stability can be achieved using lyophilization formulation for liquid fill. Filled samples were stored at -20°C and 4°C for stability study. Table 14 shows the 3-month stability data. Virus is stable at both -20°C and 4°C for the four different formulations. This is in agreement with the results from  
25 formulation set #2, which suggests that better virus stability is expected with the presence of both mannitol and sucrose in the formulation. Longer time storage stability data is being accrued.

**Liquid Formulation Set #4**

Detergents have been used in the formulations for a variety of recombinant proteins. In this set of formulation, various concentrations of detergents were examined for formulating Adp53. The detergents used were non-ionic (Tween-80) and zwitterionic (Chap). Table 15 shows the 12-month stability data. Virus is stable at 4°C storage. No significant difference in virus stability at 4°C was observed among the formulations tested. Similar to formulation set #2, no exogenous protein is included in this set of formulation.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

### REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

U.S. Patent No. 4,797,368

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**WHAT IS CLAIMED IS:**

1. A pharmaceutical adenovirus composition comprising adenovirus particles and pharmaceutical excipients, the excipients including a bulking agent and one or more protectants, wherein the excipients are included in amounts effective to provide an adenovirus composition that is storage stable.
2. The adenovirus composition of claim 1, further defined as having an infectivity of between 60 and 100% of the starting infectivity, and a residual moisture of less than about 5%, when stored for six months at 4° centigrade.
3. The adenovirus composition of claim 1, further defined as a freeze dried composition.
4. The composition of claim 3, wherein the bulking agent is further defined as a bulking agent which forms crystals during freezing.
5. The composition of claim 1, wherein the bulking agent is mannitol, inositol, lactitol, xylitol, isomaltol, sorbitol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silcate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methhylcellulose or methylcellulose.
6. The composition of claim 5, wherein said bulking agent is mannitol.
7. The composition of claim 6, further defined as an aqueous composition comprising the bulking agent in a concentration of from about 1% to about 10% (w/v).
8. The composition of claim 7, wherein the aqueous composition comprises the bulking agent in a concentration of from about 3% to 8%.

9. The composition of claim 8, wherein the aqueous composition comprises the bulking agent in a concentration of from about 5% to 7%.

5 10. The composition of claim 3, wherein the freeze dried composition was prepared from an aqueous composition comprising a bulking agent in a concentration of from about 1% to 10% (w/v).

10 11. The composition of claim 10, wherein the freeze dried composition was prepared from an aqueous composition comprising a bulking agent in a concentration of from about 3% to 8%.

15 12. The composition of claim 11, wherein the freeze dried composition was prepared from an aqueous composition comprising a bulking agent in a concentration of from about 5% to 7%.

13. The composition of claim 1, wherein said protectant is further defined as including a cryoprotectant.

20 14. The composition of claim 13, wherein said cryoprotectant is a non-reducing sugar.

15. The composition of claim 14, wherein the non-reducing sugar is sucrose or trehalose.

25 16. The composition of claim 15, wherein said cryoprotectant is sucrose.

17. The composition of claim 14, further defined as an aqueous composition comprising the non-reducing sugar in a concentration of from about 2% to about 10% (w/v).

18. The composition of claim 17, wherein the aqueous composition comprises the sugar in a concentration of from about 4% to 8%.

5 19. The composition of claim 18, wherein the aqueous composition comprises the sugar in a concentration of from about 5% to 6%.

20. The composition of claim 3, wherein the freeze dried composition was prepared from an aqueous composition comprising a non-reducing sugar in a concentration of from about 2% to 10% (w/v).

10

21. The composition of claim 20, wherein the freeze dried composition was prepared from an aqueous composition comprising a non-reducing sugar in a concentration of from about 4% to 8%.

15 22. The composition of claim 21, wherein the freeze dried composition was prepared from an aqueous composition comprising a non-reducing sugar in a concentration of from about 5% to 6%.

20 23. The composition of claim 13, wherein the cryoprotectant is niacinamide, creatinine, monosodium glutamate, dimethyl sulfoxide or sweet whey solids.

24. The composition of claim 1, wherein said protectant includes a lyoprotectant.

25 25. The composition of claim 24, wherein said lyoprotectant is human serum albumin, bovine serum albumin, PEG, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 or Tween-80.

30 26. The composition of claim 25, wherein said lyoprotectant is human serum albumin.

27. The composition of claim 24, further defined as an aqueous composition comprising the lyoprotectant in a concentration of from about 0.5% to about 5% (w/v).

5 28. The composition of claim 27, wherein the aqueous composition comprises the lyoprotectant in a concentration of from about 1% to about 4%.

29. The composition of claim 28, wherein the aqueous composition comprises the lyoprotectant in a concentration of from about 1% to about 3%.

10 30. The composition of claim 3, wherein the freeze dried composition was prepared from an aqueous composition comprising a lyoprotectant in a concentration of from about 0.5% to 5% (w/v).

15 31. The composition of claim 30, wherein the freeze dried composition was prepared from an aqueous composition comprising a lyoprotectant in a concentration of from about 1% to 4%.

20 32. The composition of claim 31, wherein the freeze dried composition was prepared from an aqueous composition comprising a lyoprotectant in a concentration of from about 1% to 3%.

33. The composition of claim 24, further defined as comprising both a lyoprotectant and a cryoprotectant.

25 34. An aqueous pharmaceutical adenovirus composition comprising a polyol in an amount effective to promote the maintenance of adenoviral infectivity.

30 35. The composition of claim 34, further defined as maintaining an infectivity of about 70% PFU/mL to about 99.9% PFU/mL of the starting infectivity when stored for six months at 4° centigrade.

36. The composition of claim 34, further defined as maintaining an infectivity of about 80% to 95% PFU/mL of the starting infectivity when stored for six months at 4° centigrade.

5

37. The composition of claim 34, wherein said polyol is glycerol, propylene glycol, polyethylene glycol, sorbitol or mannitol.

10

38. The composition of claim 34, wherein said polyol concentration is from about 5% to about 30% (w/v).

39. The composition of claim 38, wherein said polyol concentration is from about 10% to about 30%.

15

40. The composition of claim 34, wherein said polyol is glycerol, included in a concentration of from about 10% to about 30% (w/v).

20

41. The composition of claim 34, wherein said composition further comprises an excipient in addition to said polyol, wherein said excipient is inositol, lactitol, xylitol, isomaltol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silicate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methhylcellulose, methylcellulose, sucrose, dextrose, lactose, trehalose, glucose, maltose, niacinamide, creatinine, monosodium glutamate dimethyl sulfoxide, sweet whey solids, human serum albumin, bovine serum albumin, PEG, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 or Tween-80.

25

42. The composition of claim 41, wherein said composition further comprises at least a first and second of said excipients, said second excipient different from said first excipient.

30

43. A method for preparation of a long-term, storage stable adenovirus formulation, comprising the steps of:

5 (a) providing adenovirus and combining said adenovirus with a solution comprising a buffer, a bulking agent, a cryoprotectant and a lyoprotectant; and

(b) lyophilizing said solution,

10

whereby lyophilization of said solution produces a freeze-dried cake of said adenovirus formulation that retains high infectivity and low residual moisture.

44. The method of claim 43, wherein said bulking agent is mannitol, inositol, lactitol, 15 xylitol, isomaltol, sorbitol, gelatin, agar, pectin, casein, dried skim milk, dried whole milk, silcate, carboxypolymethylene, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methhylcellulose or methylcellulose.

45. The method of claim 44, wherein said bulking agent is mannitol.

20

46. The method of claim 45, wherein mannitol comprises about 0.5% to about 8% (w/v) of said formulation.

47. The method of claim 43, wherein said cryoprotectant is sucrose, dextrose, lactose, 25 trehalose, glucose, maltose, niacinamide, creatinine, monosodium glutamate dimethyl sulfoxide or sweet whey solids.

48. The method of claim 47, wherein said cryoprotectant is sucrose.



49. The method of claim 43, wherein said sucrose comprises about 2.5% to about 10% (w/v) of said formulation.

50. The method of claim 43, wherein said lyoprotectant is human serum albumin, bovine serum albumin, PEG, glycine, arginine, proline, lysine, alanine, polyvinyl pyrrolidine, polyvinyl alcohol, polydextran, maltodextrins, hydroxypropyl-beta-cyclodextrin, partially hydrolysed starches, Tween-20 or Tween-80.

51. The method of claim 50, wherein said lyoprotectant is human serum albumin.

10

52. The method of claim 43, wherein said buffer is Tris-HCl, TES, HEPES, mono-Tris, brucine tetrahydrate, EPPS, tricine, or histidine.

53. The method of claim 52, wherein said buffer is present in said formulation at a concentration at about 1 mM to 50 mM.

15

54. The method of claim 53, wherein said buffer is Tris-HCl.

55. The method of claim 54, wherein said Tris-HCl is included in a concentration of from about 1 mM to about 50 mM.

20

56. The method of claim 55, wherein said Tris-HCl is included in a concentration of from about 5 mM to about 20 mM.

57. The method of claim 43, further comprising a salt selected from the group consisting of  $MgCl_2$ ,  $MnCl_2$ ,  $CaCl_2$ ,  $ZnCl_2$ , NaCl and KCl.

25

58. The method of claim 43, wherein said lyophilizing is carried out in the presence of an inert gas.

30

59. The method of claim 43, wherein lyophilizing said solution comprises the steps of:

- 5 (a) freezing said solution;  
(b) subjecting said solution to a vacuum; and  
(c) subjecting said solution to at least a first and a second drying cycle,

whereby said second drying cycle reduces the residual moisture content of said freeze-dried cake to less than about 2%.

10

60. A method for the preparation of a long-term storage, stable adenovirus liquid formulation, comprising the steps of providing adenovirus and combining said adenovirus with a solution comprising a buffer and a polyol, whereby said adenovirus liquid formulation retains high infectivity.

15

# Lyophilization Cycle

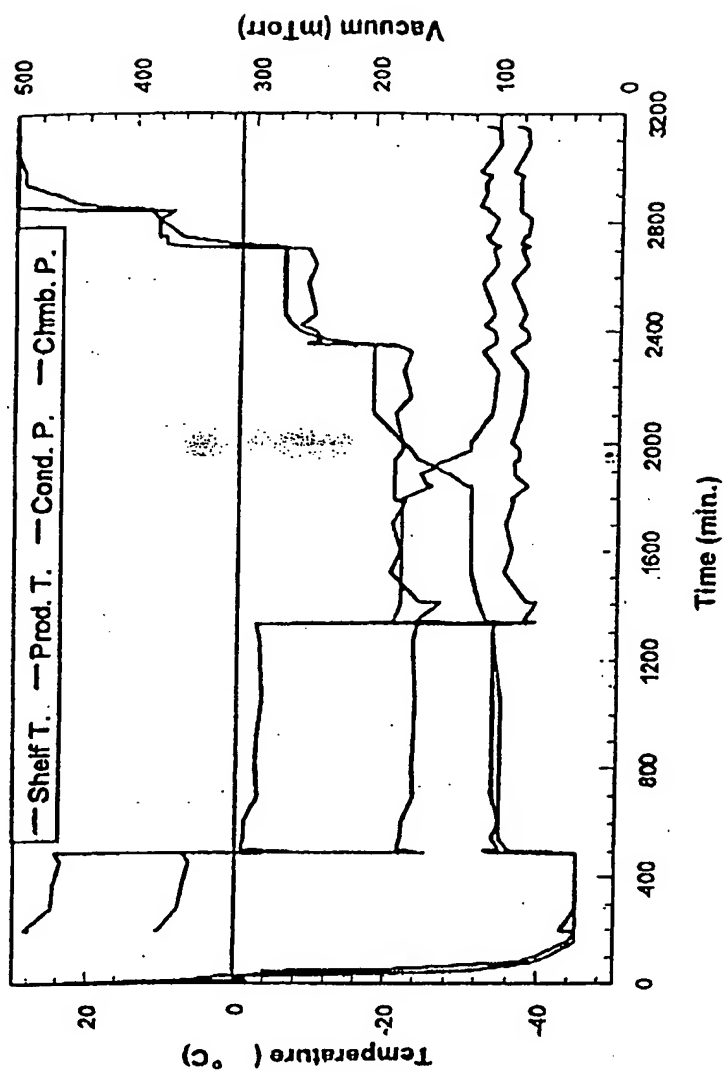


FIG. 1

# Residual Moisture of Lyophilized Adenoviral Vector during Storage

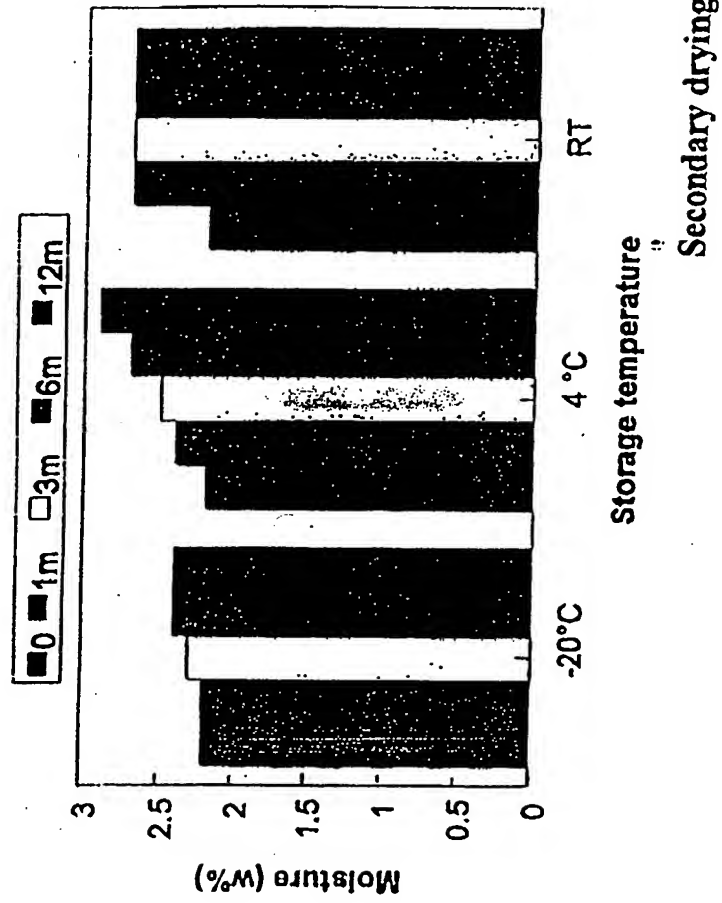


FIG. 2

### Stability of Lyophilized Adenoviral Vector during Storage

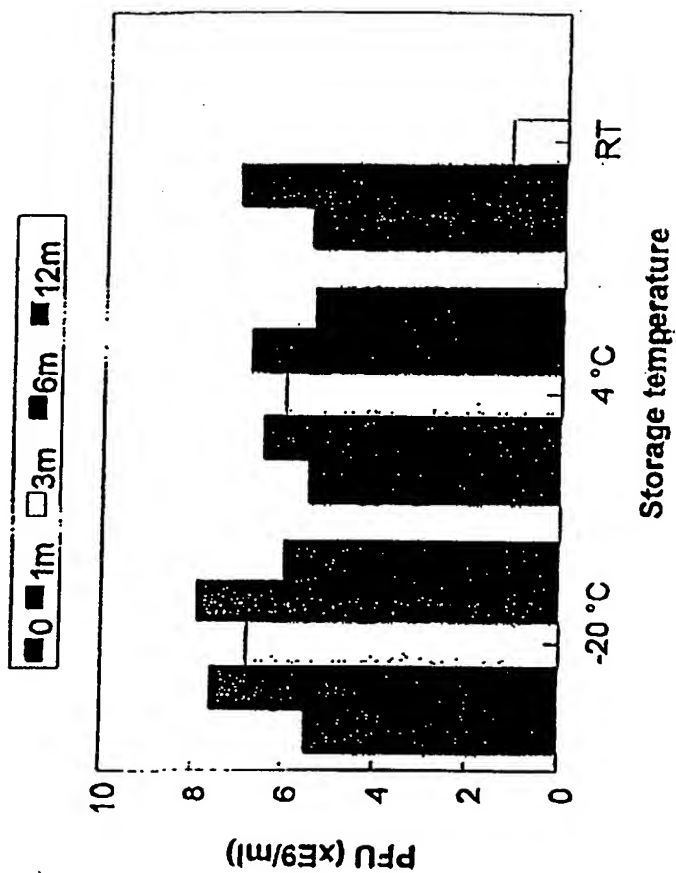


FIG. 3

# Residual Moisture of Lyophilized Adenoviral Vector during Storage

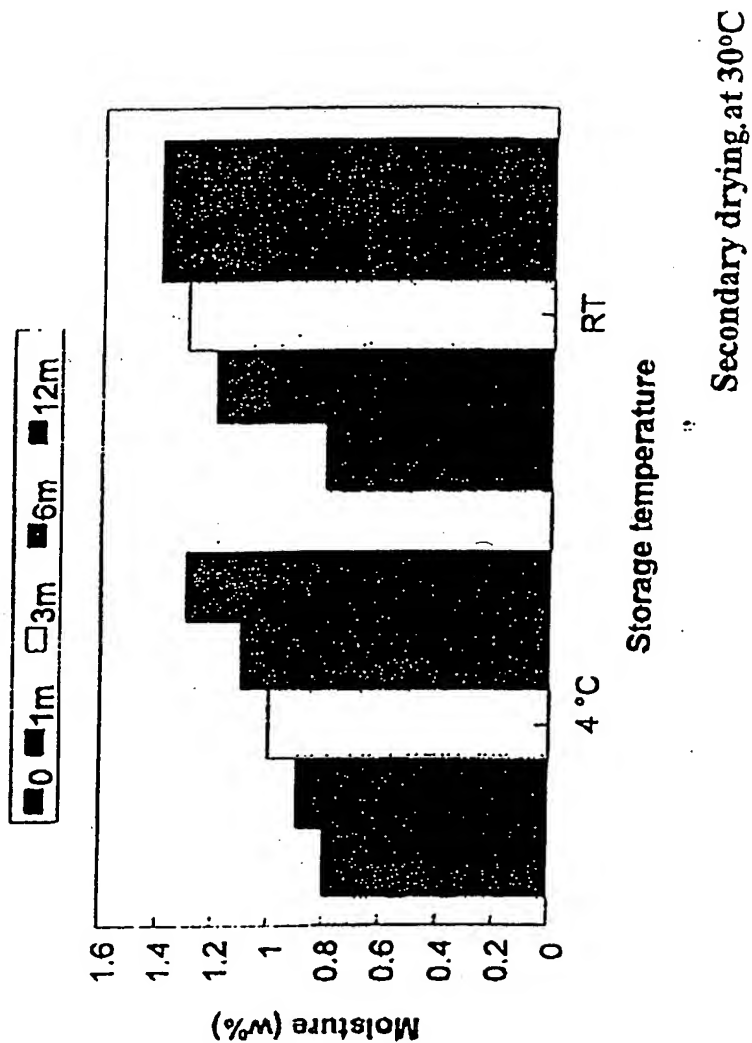
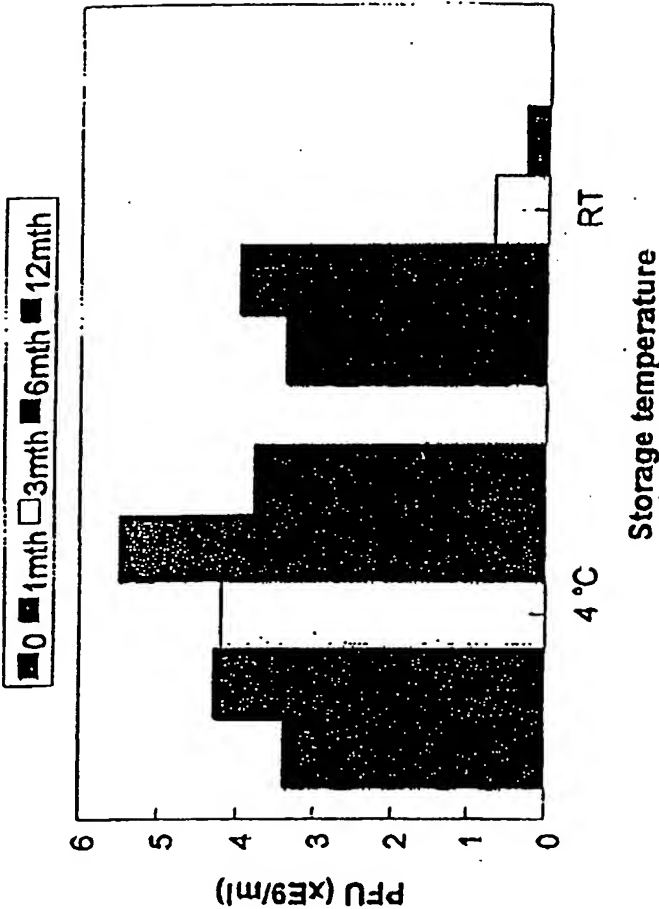


FIG. 4

Stability of Lyophilized Adenoviral Vector during Storage



Secondary drying at 30°C

FIG. 5

# HPLC Analysis of Lyophilized Adenoviral Vector Stored at RT

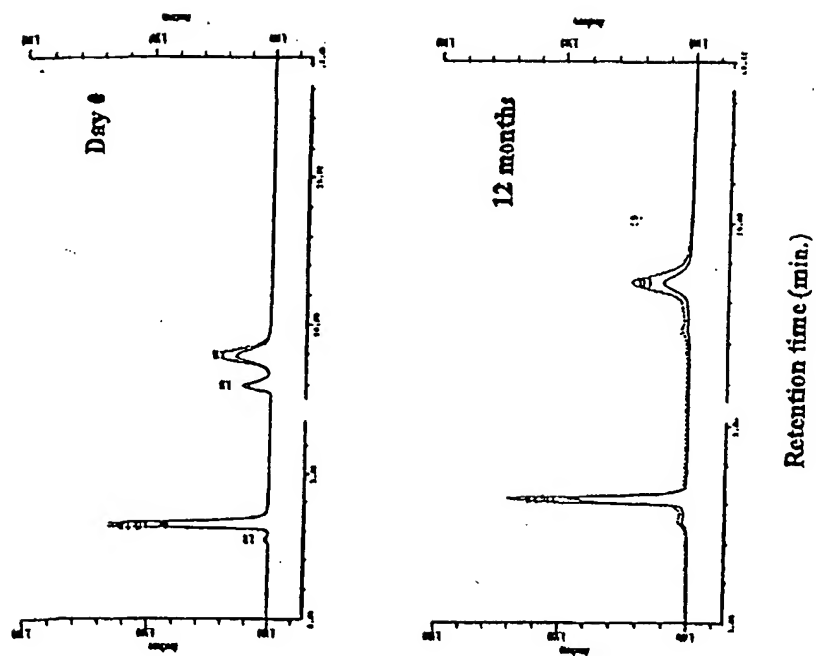
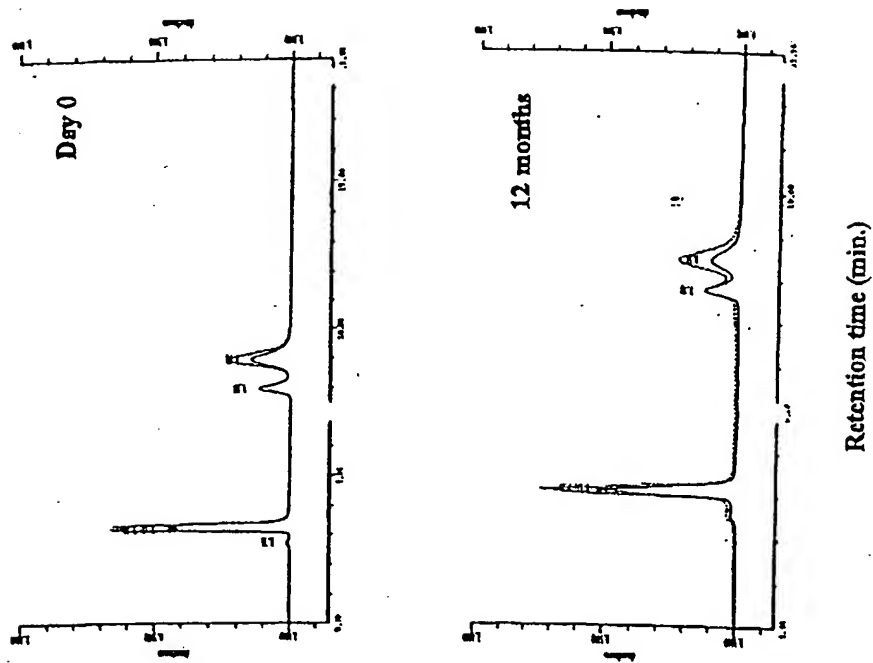


FIG. 6



# HPLC Analysis of Lyophilized Adenoviral Vector Stored at 4°C

**FIG. 7**

# HPLC Analysis of Lyophilized Adenoviral Vector Stored at -20°C

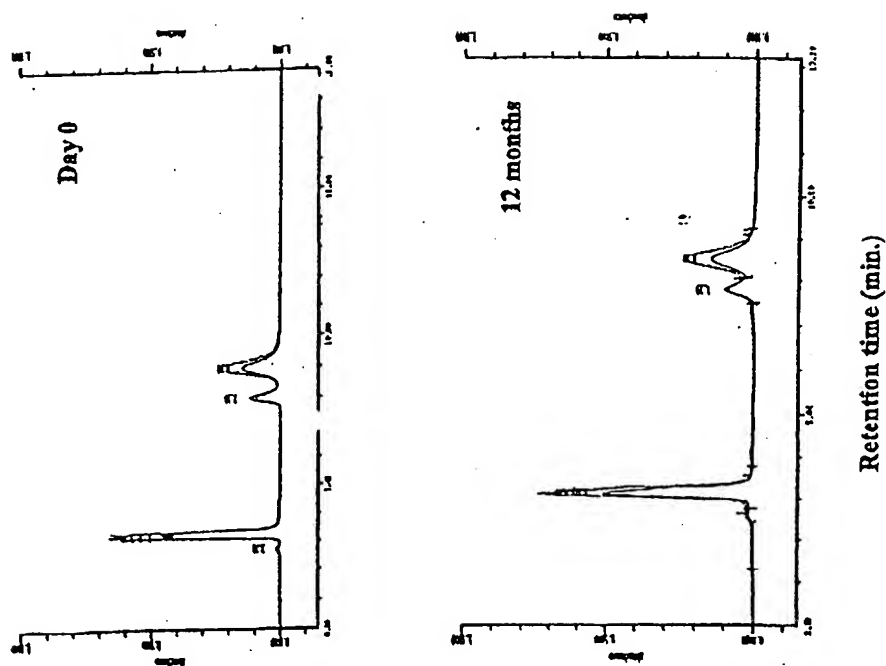
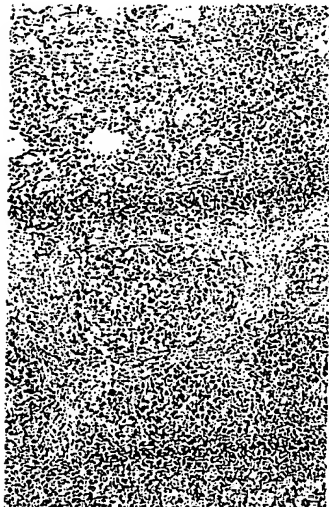


FIG. 8

**Ad- $\beta$ gal in PBS/glycerol**

**Tumor periphery**



**Tumor Center**



**Ad- $\beta$ gal in PBS/glycerol + 5% DMSO**



*Injection  $2 \times 10^{10}$  vp*



FIG. 9A

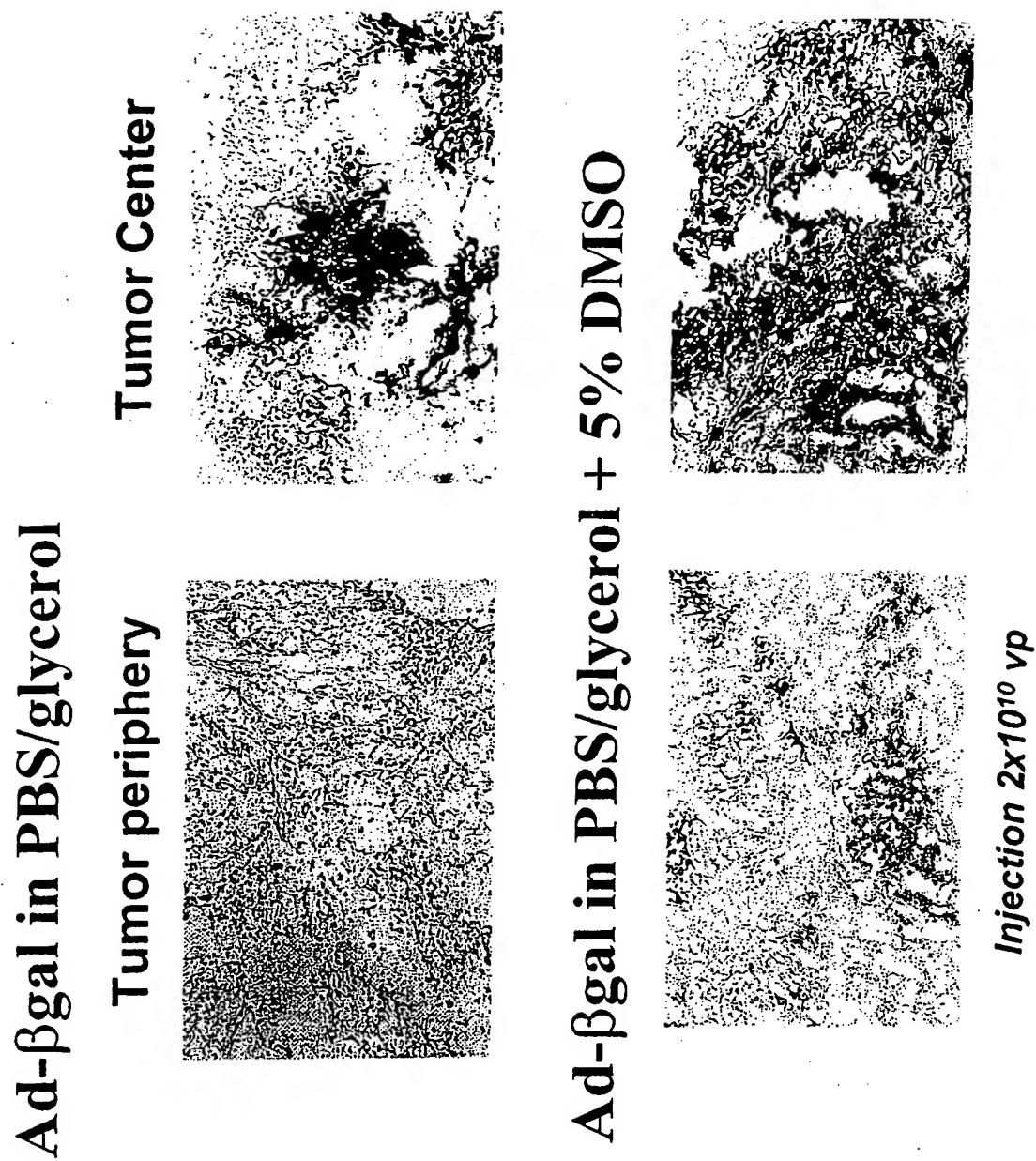


FIG. 9B

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 99/27177

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 A61K47/10 A61K47/42 A61K48/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 18790 A (CSATARY LASZLO K) 30 September 1993 (1993-09-30)  claim 5; example 3	1-4, 7, 10, 13-17, 20, 43, 47, 48, 52-57
X	WO 96 32116 A (INHALE THERAPEUTIC SYST) 17 October 1996 (1996-10-17)  page 22, line 20 -page 24, line 35	1, 2, 4-16, 24-38, 41-43, 60
X	US 5 552 309 A (MARCH KEITH L) 3 September 1996 (1996-09-03) cited in the application column 6, line 40 -column 7, line 5  -/-	34-36, 38-40, 60
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center; font-weight: bold;">10 March 2000</div>		Date of mailing of the international search report  <div style="text-align: center; font-weight: bold;">27/03/2000</div>
Name and mailing address of the ISA European Patent Office, P.B. 6818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3018		Authorized officer  <div style="text-align: center; font-weight: bold;">Seegert, K</div>

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/27177

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 22588 A (INTROGEN THERAPEUTICS INC ;CHO TOOHYON (US); ZHANG SHUYUAN (US); T) 28 May 1998 (1998-05-28) page 70, line 11 -page 72, line 9	34-37
P,X	WO 99 41416 A (SCHERING CORP) 19 August 1999 (1999-08-19) claims	34-42,60
Y	WO 95 19427 A (GENETIC THERAPY INC) 20 July 1995 (1995-07-20) Section F page 12, line 13	1-60
Y	WO 95 10601 A (VIAGENE INC) 20 April 1995 (1995-04-20) claims	1-60
Y	PATENT ABSTRACTS OF JAPAN vol. 014, no. 047 (C-0682), 29 January 1990 (1990-01-29) & JP 01 279843 A (YASUO MORITSUGU;OTHERS: 03), 10 November 1989 (1989-11-10) abstract	1-60
Y	KOTANI H ET AL: "IMPROVED METHODS OF RETROVIRAL VECTOR TRANSDUCTION AND PRODUCTION FOR GENE THERAPY" HUMAN GENE THERAPY,XX,XX, vol. 5, 1 January 1994 (1994-01-01), pages 19-28, XP000653182 ISSN: 1043-0342 "Lyophilization" page 21 page 25; table 6	1-60
P,Y	WO 99 12568 A (BURKE CARL ;MERCK & CO INC (US); VOLKIN DAVID (US)) 18 March 1999 (1999-03-18) claims	1-60
X	CROYLE M.A. ET AL: "Factors that influence stability of recombinant adenoviral preparations for human gene therapy" PHARMACEUTICAL DEVELOPMENT AND TECHNOLOGY, vol. 3, no. 3, August 1998 (1998-08), pages 373-383, XP000881617 abstract page 374; figure 1 page 378; figure 5 "Discussion" page 379 -page 381	1-12, 34-39, 41,42,60

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/27177

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WO 9519427 A	20-07-1995	CA 2181066 A	20-07-1995
		EP 0738319 A	23-10-1996
		JP 9508017 T	19-08-1997
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		US 5661022 A	26-08-1997
WO 9510601 A	20-04-1995	AU 7971194 A	04-05-1995
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JP 01279843 A	10-11-1989	JP 2042887 C	09-04-1996
		JP 7061955 B	05-07-1995
WO 9912568 A	18-03-1999	AU 9041598 A	29-03-1999

# **EXHIBIT 8**



**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:  
George H. Yoo

Serial No.: 10/747,798

Filed: December 29, 2003

For: P53 TREATMENT OF  
PAPILLOMAVIRUS AND  
CARCINOGEN-TRANSFORMED CELLS  
IN HYPERPLASTIC LESIONS

Group Art Unit: 1633

Examiner: Scott D. Priebe

Atty. Dkt. No.: INRP:104US

**DECLARATION OF LOUIS ZUMSTEIN, PhD, UNDER 37 C.F.R. §1.132**

I, Louis Zumstein, Ph.D., hereby declare as follows:

1. I have been employed by Introgen Therapeutics for over 10 years and my current position with this company is Associate Vice President of Research.

2. I have over 13 years of experience in the biotechnology field since 1993, including both research and preclinical drug development. Additionally my educational background includes a Ph.D. from Harvard University in biochemistry and molecular biology, and postdoctoral research at both Harvard University and Stanford University. A copy of my curriculum vita (or NIH biosketch) is attached as Appendix A.

3. I understand that the Examiner contends that a patent application (WO/99/66946) by El-Deiry indicates that p73 is a homolog of p53.

4. I have read the page 14, lines 5-10 of the instant specification which states:

Throughout this application, the term "p53" is intended to refer to the exemplified p53 molecules as well as all p53 homologues from other species. "Wild-type" and "mutant" p53 refer, respectively, to


a p53 gene expressing normal tumor suppressor activity and to a p53 gene lacking or having reduced suppressor activity and/or having transforming activity. Thus "mutant" p53 are not merely sequence variants but rather, are those variants showing altered functional profiles.

5. As a scientist in the biotechnology field, I feel that the aforementioned lines regarding p53 indicate that this passage is specific for human p53 and p53 in other species. I do not believe from reading this passage that "p53" as used in this passage would refer to proteins other than p53 that might share some functional characteristics with p53,.

6. Furthermore, as a scientist in the biotechnology field, it is my belief that p73 is not a homologue of p53. More specifically, while p73 and p53 do share some similar functions, and share some sequence similarities, there are important characteristics that distinguish the two proteins. For example, in contrast to p53 deficient mice, those mice lacking p73 show no increased susceptibility to spontaneous tumorigenesis. Additionally, p73 is not activated by DNA damage, unlike p53.

7. I hereby declare that all statements made by my own knowledge are true and all statements made on information and belief are believed to be true and further that statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment under § 100 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

\_\_\_\_17 February 2006\_\_\_\_\_  
Date

  
\_\_\_\_\_  
Louis Zumstein

# **APPENDIX A**

## BIOGRAPHICAL SKETCH

NAME	POSITION TITLE		
Louis A. Zumstein	Associate Vice President, Research		
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
University of Miami, FL		1973-75	
Florida State Univ., Tallahassee, FL	BS	1975-1977	Biology
Harvard University, Cambridge, MA	Ph.D.	1978-1986	Biochemistry
Stanford University, Stanford, CA	Post-doc	1987-1991	Molecular Biology
Baylor College of Medicine	Post-Doc	1991-1993	Molecular Biology

**Professional Experience:**

2/2005 to present	Associate Vice President, Research, Introgen Therapeutics, Inc., Houston, TX 77030. Supervising pre-clinical development of three gene therapy products for oncology. Overall project leader for one gene therapy product for cancer, about to start clinical development.
4/1999 - 2/2005	Director of Research, Introgen Therapeutics, Inc., Houston, TX 77030. Supervising pre-clinical development of three gene therapy products for oncology.
3/1997 - 4/1999	Associate Director of Research, Introgen Therapeutics, Inc., Houston, TX 77054. Supervise group gathering pre-clinical data on two products. Project leader for Introgen's second product through IND submission.
3/1995 - 2/1997	Research Program Manager, Vector Development, Introgen Therapeutics, Inc., Houston, TX 77054. Performed and supervised early production and process development work. Supervised clinical sample assay development, intimately involved in our Phase I HNSCC trial with RPR/INGN 201.
10/1993 - 2/1995	Assistant Director of Research, Sennes Drug Innovations, Inc., Houston, TX 77054. Supervision of production group, and cell biology/assay development group.

**Awards and Honors:**

1977	Phi Beta Kappa, Florida State University, Tallahassee, FL
1988 - 1991	NSF Postdoctoral Research Fellowship in Plant Biology

**Selected Publications:**

- Yoo GH, Piechocki MP, Oliver J, Lonardo F, Zumstein L, Lin HS, Kim H, Shibuya TY, Shehadeh N, Ensley JF. *Enhancement of Ad-p53 therapy with docetaxel in head and neck cancer*. Laryngoscope. 2004 Nov;114(11):1871-9.
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# **EXHIBIT 9**

# Monoallelically Expressed Gene Related to p53 at 1p36, a Region Frequently Deleted in Neuroblastoma and Other Human Cancers

Mourad Kaghad,\* Helene Bonnet,\* Annie Yang,<sup>†</sup>  
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Alexandre Valent,<sup>‡</sup> Adrian Minty,\* Pascale Chalon,\*  
Jean-Michel Lelias,\* Xavier Dumont,\*  
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## Summary

We describe a gene encoding p73, a protein that shares considerable homology with the tumor suppressor p53. p73 maps to 1p36, a region frequently deleted in neuroblastoma and other tumors and thought to contain multiple tumor suppressor genes. Our analysis of neuroblastoma cell lines with 1p and p73 loss of heterozygosity failed to detect coding sequence mutations in remaining p73 alleles. However, the demonstration that p73 is monoallelically expressed supports the notion that it is a candidate gene in neuroblastoma. p73 also has the potential to activate p53 target genes and to interact with p53. We propose that the dysregulation of p73 contributes to tumorigenesis and that p53-related proteins operate in a network of developmental and cell cycle controls.

## Introduction

Spontaneous lesions in the gene encoding the tumor suppressor p53 have been implicated in the progression of a wide range of human tumors (Nigro et al., 1989; Hollstein et al., 1991; Levine et al., 1995). The prevalence of tumors in individuals or mice bearing constitutional p53 mutations suggests that loss of p53 activity also contributes to the generation of tumors (Li and Fraumeni, 1969; Malkin et al., 1990; Donehower et al., 1992; Jacks et al., 1994). Wild-type p53 can also be neutralized through direct interaction with either cellular proteins or viral tumor antigens (Lane and Crawford, 1979; Linzer and Levine, 1979; Werness et al., 1990). p53 appears to induce cell cycle arrest or apoptosis in response to cellular stresses such as DNA damage and hypoxia (Kastan et al., 1992; Livingstone et al., 1992; Lowe et al., 1993; Hartwell and Kastan, 1994). By this means,

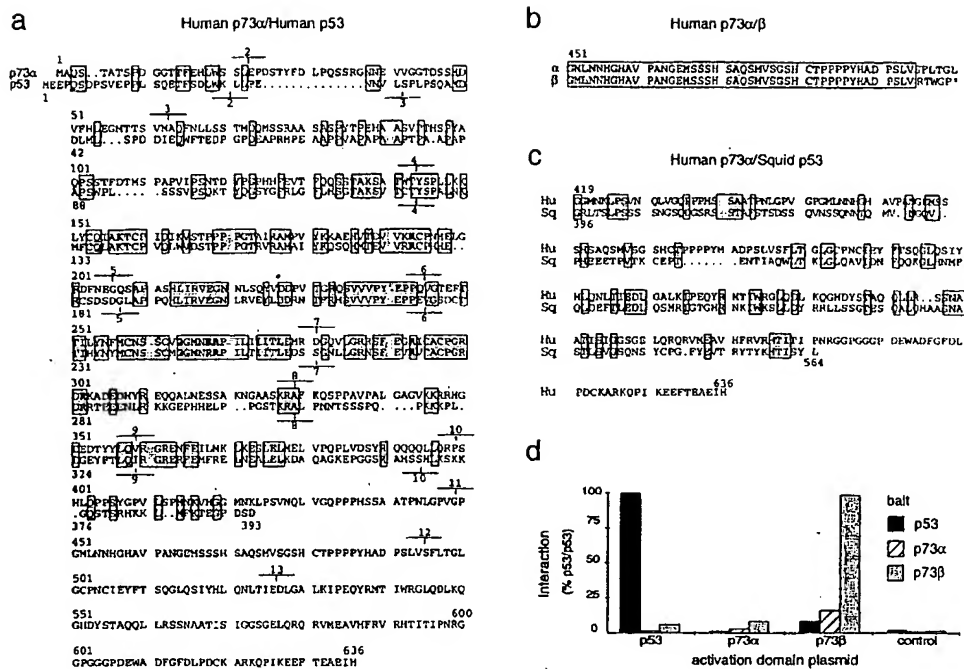
p53 acts as a tumor suppressor; its loss of function appears to confer selective advantages on cells through deregulated growth and resistance to cell death (Graeber et al., 1996; Kinzler and Vogelstein, 1996).

Despite the widespread presence of p53 mutations in human malignancies, many tumors develop in the absence of p53 abnormalities or obvious tumor antigens, most likely due to a loss of other tumor suppressor genes (Weinberg, 1993). Many of these tumor suppressor genes, including *Rb* in retinoblastoma, *NF1* in neurofibromatosis, and *DCC* and *APC* in colon carcinomas, were initially identified through cytogenetic evidence of loss of heterozygosity (LOH) (Benedict et al., 1983; Cavenee et al., 1983; Ballester et al., 1990; Buchberg et al., 1990; Fearon et al., 1987).

Similarly, extensive investigations of neuroectodermal tumors, including neuroblastoma, melanoma, and multiple endocrine neoplasm, have suggested the presence of multiple tumor suppressors at the subtelomeric region of chromosome 1 (Brodeur et al., 1977; Balaban et al., 1986; Ross et al., 1995; Sozzi et al., 1988; Dracopoli et al., 1989). Neuroblastomas with 1p LOH can be subdivided into two classes as having either small deletions (5 to 10 Mb) at 1p36.2-3 or a clinically more aggressive form characterized by *N-myc* amplification and larger deletions of chromosome 1 including subbands p36 and p35 (Brodeur et al., 1984; Takeda et al., 1994; Caron et al., 1995). Notably, the chromosome that sustains the discrete 1p36 deletion of the first class of neuroblastoma is almost exclusively of maternal origin, indicating that the putative tumor suppressor in this region is imprinted (Barlow, 1995; Caron et al., 1995). In contrast, *N-myc*-amplified neuroblastomas show a LOH at 1p35-1pter from either chromosome (Caron et al., 1995; Cheng et al., 1995). These observations suggest that neuroblastoma develops through different mechanisms of inactivating alleles of putative tumor suppressors at 1p36 and that additional genes, at 1p35 and at other loci, influence tumorigenesis. The etiology of neuroblastoma is complicated by an additional class of neuroblastoma, referred to as 4S, that initially appears as a widely disseminated, aggressive disease (Ambros et al., 1995). Remarkably, a majority of the 4S neuroblastomas suddenly and spontaneously regress in the absence of treatment. Understanding these complex pathways of neuroblastoma induction and progression will require the identification of the provisional tumor suppressors located on the short arm of chromosome 1.

We have identified a novel gene encoding a protein, termed p73, with remarkable sequence similarity to the DNA-binding, transactivation, and oligomerization domains of p53. We show that p73 has oligomerization and transactivation properties similar to p53 and that the p73 gene maps to the 1p36.33 region frequently deleted in neuroblastoma and other tumors. In addition, we provide evidence to support the notion that alterations in p73 gene expression may be one factor in the development of neuroblastoma and other tumors.

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**Figure 1. Homology between p73 and p53**

Primary amino acid sequences of human p73 $\alpha$  and  $\beta$  were deduced from cDNAs from normal human tissues.

(A) Comparative alignments of coding sequence and gene structure of human *p73α* and *p53*. Identities are enclosed in shaded boxes, with *p53* residues frequently mutated in tumors presented in bold. The identity and position of introns within *p73* and *p53* genes are denoted above and below the respective sequences.

(B) C-terminal sequence of human p73 $\beta$ , a splicing variant lacking exon 13, is aligned with its site of divergence from p73 $\alpha$ .

(C) Homology between the C-terminal sequence of p73 $\alpha$  and that of a p53-like protein from squid.

(D) Summary of yeast two-hybrid interaction assays between p53, p73 $\alpha$ , and p73 $\beta$ , presented as ordinate values relative to B-galactosidase activity of p53-p53 interactions.

## Results

### Homology between p73 and the p53 Tumor Suppressor

A cDNA encoding p73 was fortuitously discovered in a hybridization screen of a COS cell cDNA library using degenerate oligonucleotides corresponding to IRS-1-binding domains. The coding sequence of p73 was found to lack any homology to IRS-1 binding domains. Subsequently, libraries of normal human colon tissue cDNAs were screened by hybridization to yield cDNAs encoding p73 $\alpha$  and p73 $\beta$ , which are splicing variants of p73 differing at their C termini (Figures 1a and 1b). The homology between p73 and p53 is extensive within the most conserved p53 domains (Zambetti and Levine, 1993; Ko and Prives, 1996) involved with transactivation (29% identity with p53 amino acids 1–45), DNA binding (63% identity with p53 amino acids 113–290), and p53 oligomerization (38% identity in p53 sequence from 319–363) (Figures 1a and 1c). While the homology between the N terminus of p73 and that required for transcriptional activation by p53 is not strong, a sequence similar to the MDM2-binding domain of p53 (TFSDLW; Lin et al., 1994a) is present in p73 as TFEDLW. Significantly, residues corresponding to those of p53 frequently mutated in tumors (R175, G245, R248, R249, R273, and R282) and shown to be required for sequence-specific DNA recognition (Lin et al., 1994b; Ko and

Prives, 1996) are conserved and occupy identical positions in p73 (Figure 1a). No significant homology was detected between the C-terminal domain (364–393) of mammalian p53 and p73. However, the C-terminal domain of human p73 $\alpha$  shows homology with recently discovered invertebrate p53 homologs (Figure 1c), suggesting the possibility that *p53* may have evolved from a more primitive, *p73*-like gene. In support of this concept, the intron–exon organization of the *p73* gene was found to be similar to that of the *p53* gene (Figure 1a).

p73 $\beta$  is encoded by transcripts lacking the 96 nucleotides corresponding to exon 13. This deletion interrupts the open reading frame, yielding a polypeptide of 499 amino acids (Figure 1b). Both p73 $\alpha$  and  $\beta$  transcripts were detected by PCR in all human tissues tested, including brain, kidney, placenta, colon, heart, liver, spleen, and skeletal muscle (data not shown), indicating a widespread, albeit low level, expression of these proteins.

Considering the extensive homology between p53 and p73, including that in the oligomerization domain of p53, we assessed whether these proteins would interact in the context of the yeast two-hybrid system (Gyuris et al., 1993). Using this assay, we detected strong homotypic interactions between p53 molecules, indicative of their known ability to form oligomers (Ko and Prives, 1996). In contrast, p73 $\alpha$  showed a very low tendency to form homotypic interactions in this assay. However,



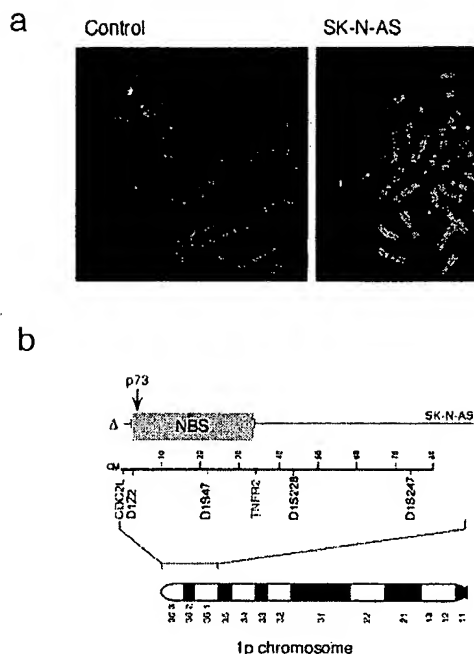


Figure 2. Mapping of *p73* Gene to 1p36 and LOH in Neuroblastoma Cell Line

(A) (Left panel) Fluorescence in situ hybridization (FISH) of a cosmid probe containing the *p73* gene on a normal human chromosome spread. Chromosomes (in red) are counterstained with propidium iodide, and *p73* gene signals appear as green at the telomere of 1p. (Right panel) FISH analysis on chromosome spreads of the SK-N-AS neuroblastoma cell line reveals two chromosome 1 homologs using the centromeric D1Z5 probe (red) but only one chromosome 1 with a *p73* signal (green). Chromosomes are counterstained with DAPI (blue).

(B) Summary of cytogenetic data for the localization of the neuroblastoma suppressor locus at 1p36, based on overlapping regions of deletion in neuroblastoma cell lines and detailed mapping of the 1p deletion in the SK-N-AS cell line (Cheng et al., 1996). Approximate positions of genes (in red) and polymorphic markers (in black) are noted.

*p73β* displayed strong homotypic interactions, equivalent to that of *p53*. The potential for heterotypic interactions was also assayed by the two-hybrid system. Interestingly, *p53* and *p73β* displayed significant mutual interactions in either bait or prey configuration, while *p53* showed negligible interactions with *p73α*. Weak but detectable interactions between *p73α* and *p73β* were also evident in this assay. The physiological significance of the apparent oligomerization and restricted heterotypic interactions amongst *p73α*, *p73β*, and *p53* determined in these assays remains to be established.

#### *p73* Gene Localized to Neuroblastoma Suppressor Locus at 1p36

To test the possibility that the *p73* gene was located at sites of suspected tumor suppressor genes, we mapped the *p73* gene using fluorescence in situ hybridization (FISH) on normal human chromosome spreads. The *p73* cosmid probe hybridized to the subtelomeric p36 region of chromosome 1 (Figure 2a). As deletions in the short

arm of chromosome 1 appear frequently in neuroblastoma (Takeda et al., 1994; Caron et al., 1995; Cheng et al., 1995), we performed *p73* FISH analysis on various human cell lines established from neuroblastoma tumors. The SK-N-AS has the smallest 1p deletion of defined neuroblastoma cell lines covering approximately 8 Mb, limited distally by D1Z2 (1p36.33) and proximally by *TNFR2* (1p36.2) (Figure 2b; White et al., 1995; Cheng et al., 1996). Probing of SK-N-AS with the *p73* gene yielded signal from only one of the two chromosome 1 homologs, demonstrating a *p73* LOH in this neuroblastoma cell line (Figure 2a). Subsequent, more detailed mapping has indicated that the *p73* gene is very close to the D1Z2 marker at 1p36.33, thereby placing *p73* at the distal border of the consensus region of deletion in neuroblastoma (Figure 2b).

A similar FISH analysis of neuroblastoma cell lines showing *N-Myc* amplification and a larger 1p deletion, including IMR-32 and CHP-212, confirmed the LOH of the *p73* gene in these cells (Table 1). Interestingly, both the SK-N-SH and the SK-N-MC neuroblastoma cell lines show neither 1p (Davidoff et al., 1992; Cheng et al., 1996) nor *p73* LOH (Table 1), indicating that either other lesions are responsible for these tumors or that *p73* is inactivated by mechanisms besides deletions in these cell lines.

#### *p73* Expression in Neuroblastoma Cell Lines

To examine *p73* and *p53* expression in neuroblastoma cell lines, we developed sensitive RT-PCR reactions to yield amplicons corresponding to the entire coding sequences of *p73* and *p53* transcripts. RT-PCR products of *p73* transcripts were first obtained from IMR-32, SK-N-MC, SK-N-SH neuroblastoma cell lines, as well as the HT-29 colon carcinoma cell line, but not from the SK-N-AS cell line (Figure 3a). To gain quantitative information on levels of *p73* transcript expression, Northern analysis was performed on these same cell lines. Two *p73* transcripts of 4.4 and 2.9 kb, corresponding to polyadenylation variants as determined by direct sequencing, were detected in HT-29, IMR-32, and SK-N-SH mRNA, while *p73* transcripts were either not detected in RNA from SK-N-AS cells or present at exceeding low levels in SK-N-MC cells (Figure 3b, upper panel). Corresponding Northern analysis of *p53* transcripts in these cell lines revealed a common 2.5 kb transcript except for SK-N-MC, which displays a previously characterized truncation (Figure 3b, lower panel; Davidoff et al., 1992). Western blots to detect the *p73α* protein were performed using a polyclonal antibody directed against the C terminus of *p73α*. Reflecting the Northern data for *p73* transcripts in these cell lines, extracts from HT-29, IMR-32, and SK-N-SH cells showed easily detectable levels of the *p73α* protein. However, SK-N-MC and SK-N-AS extracts contained significantly reduced levels of *p73α*, showing only a faint band that migrates somewhat faster than the main product seen for HT-29, IMR-32, and SK-N-SH (Figure 3c). To characterize further the expression pattern of *p73*, we performed immunolocalization of the endogenous *p73α* in an array of cell lines, all of which revealed a pattern of small, punctate dots in the nucleus of some cells in an asynchronous

Table 1. Analysis of p73 in Neuroblastoma and Other Tumor Cell Lines

Cell Lines	1pΔ <sup>1</sup>	Gene Copy # <sup>2</sup>	D1Z5 Copy # <sup>3</sup>	Allele(s) <sup>4</sup>	Expressed Allele <sup>5</sup>	Transcript Sequence <sup>6</sup>	Protein <sup>7</sup>	p53 Transcript <sup>8</sup>
<b>Neuroblastomas</b>								
(N-Myc amplified)								
IMR-32	Yes	1	3	A/T; (-)	A/T	wt	+	wt
CHP-212	Yes	1	3	A/T; (-)	A/T*	wt	-	wt
SMS-BC	Yes	NT	NT	G/C; (-)	G/C*	wt	-	mut [135C→F]
SK-N-BE(2)	Yes	NT	NT	G/C; (-)	G/C	wt	-	mut [135C→F]
SMS-KAN	Yes	NT	NT	G/C; (-)	G/C*	wt	-	wt
(N-Myc single copy)								
SK-N-AS	Yes	1	2	G/C; (-)	G/C*	wt	-	wt
SK-N-MC	No	2	2	G/C; G/C	G/C	wt/mut [472A→T]	-	Δexons2-4
SK-N-SH	No	2	2	G/C; A/T	A/T	wt/Δexon2 <sup>8</sup>	+	wt
<b>Others</b>								
NCI-1011	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [176C→F]
HT-29	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [273R→H]
A-172	NR	NT	NT	G/C; (?)	G/C	wt	+	wt
U-373	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [273R→H]
K-562	NR	NT	NT	G/C; (?)	G/C	wt	+	frame shift
IM-9	NR	NT	NT	G/C; (?)	G/C	wt	+	wt
SAOS-2	NR	3	5	G/C; G/C	G/C	wt	-	(-)
MCF-7	NR	3	2	G/C; G/C	G/C	wt	+	wt
U-937	NR	3	4	G/C; G/C	(-)	N/A	-	(-)
HL-60	NR	2	2	G/C; G/C	(-)	N/A	-	(-)
SW-480	NR	NT	NT	G/C; (?)	G/C	wt	+	mut [273R→H] mut [309P→S]

<sup>1</sup>1pΔ, deletion in short arm of chromosome 1, as cited in text. NR, none reported.<sup>2</sup>Determined by fluorescence in situ hybridization (FISH) with a p73 genomic probe on chromosome spreads, performed as described in Experimental Procedures. NT, not tested.<sup>3</sup>Determined by FISH analysis with the chromosome 1 centromeric probe, D1Z5 (Oncor). NT, not tested.<sup>4</sup>Refers to the polymorphism of G/C and A/T alleles, as determined by PCR on genomic DNA and PCR-RL (see Figure 4 and Experimental Procedures) of products. Minus sign (-) indicates p73 loss of heterozygosity, assumed on the basis of known 1p36 deletions. For cell lines in which chromosome 1p deletions have not been reported (NR), the A/T allele was never detected, though the presence of other non-G/C or A/T alleles cannot be formally excluded (denoted by question mark).<sup>5</sup>Determined by RT-PCR on mRNA and subsequent sequencing of products. Asterisks indicate a second, 30-cycle round of PCR was required to generate products for sequencing. (-) indicate a lack of detectable product, despite two rounds of RT-PCR.<sup>6</sup>Determined by sequence analysis of open reading frame in expressed transcripts. wt, wild type compared with normal human tissue (not shown); N/A, not available due to lack of mRNA expression, as determined by RT-PCR. δ indicates 10% of transcripts sequenced contained a deletion in exon 2.<sup>7</sup>Detectable by Western blotting of whole cell lysates, as described in Figure 3 and Experimental Procedures.<sup>8</sup>Same as 6, performed on p53 mRNA.

population (Figure 3d). A similar distribution of dots was revealed upon transfecting mammalian cells with a myc epitope-tagged p73α expression vector, indicating that p73 autonomously targets to these intranuclear foci (Figure 3d).

#### Lack of Coding Region Mutations in p73 Gene

As most neuroblastoma cell lines examined displayed a LOH of the p73 gene, we asked whether the remaining allele sustained genetic changes that might affect p73 function. We used the p73 RT-PCR reaction to amplify the coding region of p73 from mRNA of an extended group of neuroblastoma cell lines. As with SK-N-AS, the neuroblastoma lines SMS-BC, CHP-212, and SMS-KAN showed no RT-PCR product corresponding to p73 after 30 cycles, suggesting very low expression levels. However, reamplification for an additional 30 cycles yielded sufficient product for sequencing p73 transcripts from these lines. These PCR products, including six from neuroblastoma cell lines displaying a 1p36 LOH (IMR-32, CHP-212, SMS-BC, SMS-KAN, SK-N-BE(2), and SK-N-AS), two from neuroblastoma cell lines that showed no 1p deletion (SK-N-SH and SK-N-MC), as well as those

from control, nonneuroblastoma lines, were sequenced in their entirety and found to be identical to p73 sequences derived from normal human tissue (Table 1). Western blot analysis of the four neuroblastoma lines that showed extremely low levels of p73 transcript by RT-PCR revealed a corresponding lack of p73α protein (Table 1). In addition, two neuroblastoma lines, SK-N-BE(2) and SK-N-MC, showed detectable levels of p73 transcripts by one round of PCR but no p73α protein. These results, together with the absence of mutations in the remaining allele of p73 LOH neuroblastoma cell lines, suggest that epigenetic mechanisms such as imprinting and translational suppression act to limit p73 expression in neuroblastoma cells.

#### Monoallelic Expression of the p73 Gene

Through our analysis of p73 transcripts in neuroblastoma cell lines and tumors, we discovered an allelic polymorphism consisting of a double nucleotide substitution (G→A) and (C→T) at positions 4 and 14 of exon 2, just upstream of the initial AUG of p73 (Figure 4a). We denote these two naturally occurring p73 alleles G/C and A/T. The G/C, A/T polymorphism occurs in a region

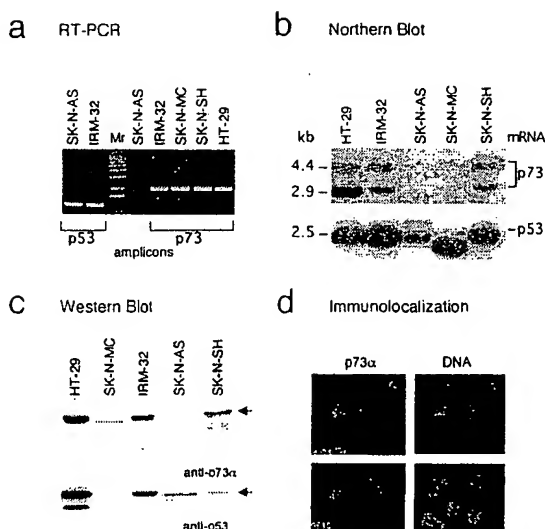


Figure 3. Analysis of *p73* Expression in Cell Lines

(A) RT-PCR products corresponding to the coding regions of *p53* and *p73α* from various cell lines used for sequence analysis (see text).

(B) Northern blot analysis of *p73* and *p53* transcripts in selected cell lines. The 4.4 and 2.9 kb *p73* transcripts result from the use of distinct polyadenylation sites. All cell lines show a similar 2.5 kb *p53* transcript except SK-N-MC, which contains a previously characterized deletion (Davidoff et al., 1992).

(C) Western blot analysis of total cell lysates cell lines using polyclonal antibody directed to Cterminus of *p73α* (top) and monoclonal antibody to *p53* (bottom).

(D) Immunolocalization of endogenous and transfected *p73α* in cell lines. (Top left panel) Immunolocalization of endogenous *p73α* in U251 human glioblastoma cell line using anti-*p73α* antibodies, revealing numerous discrete foci with the nucleus of some, but not all cells in field. (Top right panel) Hoechst dye staining of field corresponding to left panel, revealing nuclei of cells. (Bottom left panel) Immunolocalization of myc epitope-tagged *p73α* in baby hamster kidney cells transfected with a *p73α* expression vector. (Bottom right panel) Hoechst dye stained nuclei corresponding to field presented at left.

of the transcript that could theoretically form a stem-loop structure, possibly an indication of regulatory function. Interestingly, sequencing of *p73* transcripts from a wide variety of nonneuroblastoma cell lines, listed in Table 1, revealed only the G/C allele, while three of eight neuroblastoma lines possessed an A/T allele. We used PCR-restriction length analysis (PCR-RL) to screen rapidly for allele types at both the DNA and transcript levels, taking advantage of the additional StyI site resulting from the double substitution (G→A and C→T) in exon 2 (Figures 4b and 4c). Notably, the single remaining allele of both IMR-32 and CHP-212 bears the A/T polymorphism (Table 1). We were especially intrigued by the analysis of the SK-N-SH cell line, which shows no *p73* LOH and has both G/C and A/T alleles at the genomic level. At the transcript level, however, using RT-PCR-RL, we could only detect expression of the A/T allele (Figure 4c). This result suggested that, at least in SK-N-SH cells, *p73* protein is a consequence of monoallelic expression. Another possibility that might explain this result is that our RT-PCR-RL assay may favor one allele

over the other. We addressed this issue by performing the assay on G/C and A/T transcripts mixed at known ratios and showed that this assay was not obviously biased for one allele. Moreover, this analysis showed that we could detect both transcripts even when they are present in ratios of 20:1 (Figure 4d). To examine further the possibility of monoallelic expression of *p73*, we performed PCR-RL on transcripts of peripheral blood cells from five healthy donors determined to be G/C:A/T heterozygotes. All five PCR-RL assays revealed a *p73* pattern corresponding to either, but not both, the A/T or the G/C allele, supporting the notion of monoallelic expression of *p73*, at least within the 20-fold sensitivity of this assay (Figures 4c and 4d). Thus, monoallelic expression of *p73* may have particular significance for neuroblastoma and other tumors that display 1p36 LOH, as deletion of the active allele may result in a nearly complete loss of *p73* activity. Finally, *p73α* protein expression was easily detected in a majority of nonneuroblastoma cell lines. In contrast, only two of eight neuroblastoma cell lines yielded *p73α* signal on Western blots, and these exclusively express the A/T transcript (Table 1).

#### *p21<sup>ras</sup>* Induction and Growth Suppression by *p73*

The SK-N-AS cell line expresses no detectable *p73* protein and negligible levels of *p73* transcript and therefore represented an ideal model for testing the effect of reintroducing *p73* (Baker et al., 1990). SK-N-AS cells were transfected with a plasmid expressing both the selectable marker for neomycin resistance (Neo<sup>r</sup>) and either wild-type *p73α* or *p73α*(R292H), a mutant version homologous with *p53*(R273H) that is defective for DNA binding and transcriptional activation (Lin et al., 1994a; Ko and Prives, 1996). Wild-type *p53* or *p53*(V143A) (Baker et al., 1990) were transfected separately and used for comparison with *p73* samples. Cells were grown in the presence of G418, a neomycin analog, and one set of plates harvested after 48 hrs for Western blotting analysis. Lysates from the transfected cells were probed with antibodies to detect the expression of *p73* or *p53* as well as *p21<sup>ras</sup>*, a known *p53* target gene (El-Deiry et al., 1993). Significantly, cells expressing wild-type *p73* showed elevated levels of *p21* protein, comparable to those seen in wild-type *p53* transfectants, whereas mutant *p73*- and mutant *p53*-expressing cells both failed to show a similar *p21<sup>ras</sup>* induction (Figure 5a).

We further tested the effect of exogenously expressed *p73* on SK-N-AS cells using a standard colony assay (Baker et al., 1990). Identical sets of plates to those above were maintained under G418 selection for 3 weeks and assayed for colony production. No colonies were obtained from cells expressing wild-type *p73* or *p53* (Figure 5b), while the DNA-binding domain mutants of each yielded high numbers of colonies. Although the physiological significance of colony assays in general is unclear, the obvious distinction between the wild-type versions of *p73* and *p53* and their counterpart mutants that fail to bind DNA suggests that the observed growth suppression and endogenous *p21* activation in SK-N-AS cells are a function of the transcription activity of *p73* and *p53*.

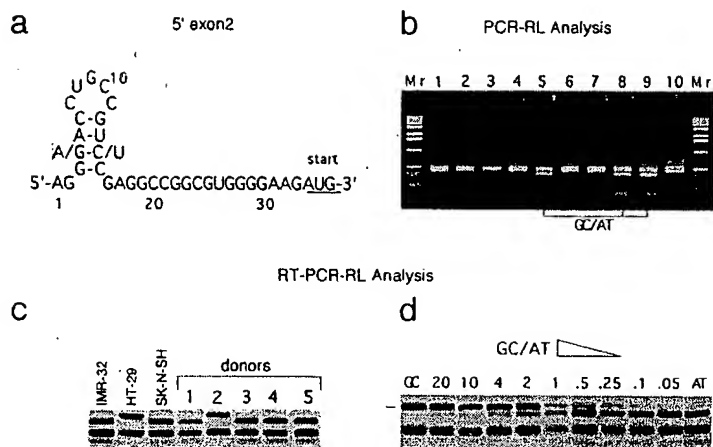


Figure 4. Monoallelic Expression of *p73* Gene

(A) Sequence of exon 2 allelic variants of *p73α* transcripts deduced from RT-PCR product sequencing. The two alleles, which represent dinucleotide substitutions at position 4 and 14 of exon 2, are G4/C14 and A4/T14 and appear at a region of the 5'-untranslated sequence that might form a stem-loop structure.

(B) Genotypic analysis of *p73* G/C and A/T alleles in ten normal blood donors using PCR-RL. Genomic PCR products that include exon 2 yield a 482 bp fragment. Sty1 digestion of PCR products yields two smaller size fragments from AT allele-derived amplicons of 376 bp and 106 bp, respectively, whereas the GC allele-derived amplicons remain uncut (lanes 1, 2, 3, 4, 6, 7, and 10). The lanes 5, 8, and 9 show heterozygotes GC/AT donors.

(C) Analysis of G/C A/T *p73* allele expression using Sty1 and Nar1 digestions of transcript RT-PCR products from cell lines and five *p73* heterozygote GC/AT individuals (1-5). As with the genomic analysis, the double digestion of RT-PCR products yields specific fragments identifying each type of allele transcript-derived amplicons (284 bp for GC and 234 bp and 50 bp for AT transcripts). IMR-32, SK-N-SH, as well as blood cells of individuals 1, 3, 4, and 5 predominantly express the A/T allele at the transcript level, while HT-29 and individual 2 express the G/C allele in transcripts.

(D) A/T G/C titration assay to determine sensitivity and bias potential of RT-PCR-RL analysis for allelic expression. G/C and A/T transcripts from total mRNA of HT-29 and IMR-32 respectively were quantified by Northern blots and mixed at the ratios indicated and analyzed by the RT-PCR-RL assay described for (C). A/T and G/C alleles can be detected even when mixed with a 20-fold excess of the other allele.

### *p73* Is Not Activated by Actinomycin D or UV Irradiation

In light of the structural similarities between *p73* and *p53*, we asked whether *p73*, like *p53*, is induced by agents that activate the DNA damage checkpoint (Ko and Prives, 1996). To do this, *p53* and *p73* protein levels were assayed in IMR-32 cells following exposure to either actinomycin D or ultraviolet radiation (Kessis et al., 1993; Caelles et al., 1994). Actinomycin D at low concentrations (1 nM) activates the DNA damage checkpoint through producing DNA strand breaks, while at higher concentrations (1 to 2 μM) inhibits transcription (Kessis et al., 1993; Caelles et al., 1994). After 24 hrs of treatment with 1 nM actinomycin D, *p53* and *p21<sup>waf</sup>* levels in the cell are markedly elevated, while *p73α* levels appear unaffected (Figure 6a). At micromolar concentrations of actinomycin D that inhibit transcription, *p53* levels continue to rise above those of untreated cells, presumably due to a stabilization of the *p53* protein. In contrast, *p21* and *p73α* protein levels were not enhanced by actinomycin D over those of untreated cells (Figure 6a). Similar results were obtained with other cell lines.

To assess further the effect of DNA damage on *p73α* levels, IMR-32 cells were exposed to ultraviolet (254 nm) radiation and subsequently probed with antibodies to *p73α*, *p53*, and *p21*. Although *p53* protein was markedly increased in cells at 15 hrs after irradiation, *p73α* levels fail to show a similar increase after such treatments (Figure 6b). A similar failure to induce *p73α* was observed in cells exposed to gamma radiation or genotoxic agents such as doxorubicin (data not shown). Thus, despite the structural similarities between *p73* and *p53* and their common ability to induce *p21<sup>waf</sup>*, these proteins do not respond in a similar manner to DNA damaging events.

### Discussion

The identification of a novel gene located at chromosome 1p36.2-3 that encodes proteins with significant homology to *p53* may have implications for our understanding of the etiology of neuroblastoma and other tumors as well as for *p53* evolution and function. The remarkable homology between the core domain of *p73* and the DNA-binding domain of *p53*, together with *p73*'s ability to induce the *p21<sup>waf</sup>* protein, suggest that *p73* acts, in part, as a transcription factor. While it is not obvious that *p73* and *p53* share common functions, the presence of distal 1p alterations in a wide array of tumors in addition to neuroblastoma, including melanoma (Dracopoli et al., 1989), hepatocellular carcinoma (Yeh et al., 1994), and ductile breast carcinoma (Genuardi et al., 1989), supports the notion that *p73* operates in pathways that coordinate cell growth, death, and differentiation.

### Neuroblastoma: LOH and Monoallelic Expression of *p73* Gene

Neuroblastoma is thought to arise from primitive neuroectodermal stem cells that fail, at various stages, to differentiate into sympathetic neurons, Schwann cells, or melanocytes (Knudson and Meadows, 1980; Ross et al., 1995). Associated cytogenetic characteristics include discrete and gross deletions of the short arm of chromosome 1, the amplification of *N-Myc*, and disturbances in cell ploidy (Takeda et al., 1994; Ambros et al., 1995; Caron et al., 1995; Cheng et al., 1995). Genetic analyses of neuroblastoma tumors harboring discrete 1p36.2-3 LOH have shown this deletion to be sustained predominantly by the maternally derived chromosome, thereby implicating one or more imprinted tumor suppressor genes in this region (Versteeg et al., 1995). In

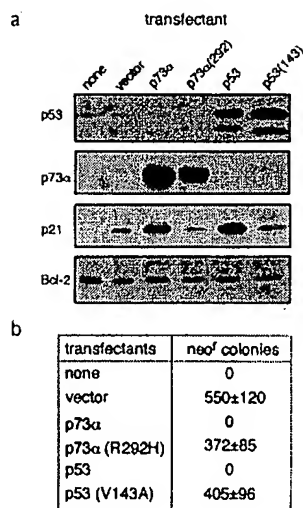


Figure 5. Expression of Exogenous p73 and p53 in SK-N-AS cells (A) SK-N-AS cells were transfected with vectors expressing Neo' alone or together with p73α, the p73α mutant (R292H), p53, or p53(V143A), grown for 48 hr, and analyzed by Western blot using antibodies to p53, p73, p21<sup>waf</sup>, and Bcl-2. (B) Colony formation in SK-N-AS cells transfected as in (A) after growth for 3 weeks under G418 selection.

contrast, the more aggressive stage 3 and 4 neuroblastomas appear to have amplified *N-Myc* and have sustained larger telomeric deletions, including 1p36 and 1p35 from chromosome 1 of random parental origin (Cheng et al., 1995). Whether 1p35 harbors additional tumor suppressor genes or one that affects *N-Myc* amplification or modifies expression of other genes on 1p is unknown, but it is obvious that neuroblastoma is a highly complex and heterogeneous disease involving the disruption of activities at multiple loci.

The chromosomal localization and monoallelic expression of *p73*, its frequent LOH in neuroblastoma, and its conspicuous lack of expression in a majority of neuroblastoma cell lines are all consistent with the notion that *p73* is a candidate for the putative, imprinted neuroblastoma suppressor gene at 1p36. Importantly, *p73* maps to the distal border of the consensus deletion found in neuroblastoma, as defined by a wide range of tumor cell lines (Cheng et al., 1996). It is interesting to note, however, that a constitutional 1p36 deletion in one case reportedly retained the D1Z2 marker at 1p36.33 (Biegel et al., 1993). This potentially places *p73* immediately distal to the region of overlap between the constitutional case and deletions in neuroblastoma tumor cell lines. It remains possible, nonetheless, that the constitutional deletion encompasses a region extremely close to *p73* and could mediate its disruption without a strict deletion of the gene. On the other hand, various studies have reported tumor cell lines and constitutional translocations at 1p36 whose break points do not map within the deletion in SK-N-AS. This has led to the speculation that multiple neuroblastoma suppressor genes exist at 1p36 (Takeda et al., 1994; Amler et al., 1995; Laureys et

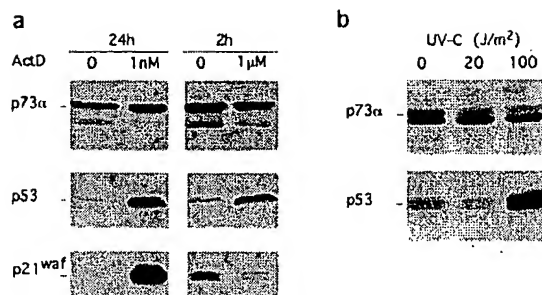


Figure 6. *p73* Induces *p21* but Is Not Responsive to DNA Damage (A) Immunoblots of lysates prepared from IMR-32 cells treated with either 1 nM actinomycin D for 24 hrs or 1 μM actinomycin D for 2 hrs were probed with antibodies to p73α, p53, and p21<sup>waf</sup>. (B) Immunoblots of lysates prepared from IMR-32 cells grown for 15 hrs after 254 nm UV-C irradiation (20 or 100 Joules/m²) probed with antibodies to p73α and p53.

al., 1995; Versteeg et al., 1995). The disruption of *p73*, then, may be one in a number of events that contribute to the onset and progression of neuroblastoma.

While the majority of neuroblastoma cell lines analyzed here lacked *p73* expression, we were able to detect protein in two exceptions: the SK-N-SH cell line, which lacks detectable 1p or *p73* LOH, and IMR-32, which harbors a large 1p35-36 deletion characteristic of *N-myc*-amplified neuroblastomas. Notably, sequence analysis of *p73* transcripts in both SK-N-SH and IMR-32 revealed a dinucleotide A/T polymorphism in the 5' untranslated region. Using this polymorphism, we demonstrated monoallelic expression of the *p73* gene not only in cell lines but also in peripheral blood cells of normal donors. To date, we have analyzed only one informative family regarding the parental dominance in expression of *p73* alleles. In this case, a normal A/T/G/C heterozygote donor was found to express the A/T allele at the transcript level, while the maternal and paternal genotypes were A/T;A/T and G/C;G/C, respectively (data not shown). While obviously limited, this analysis shows a case in which the *p73* gene, like the putative tumor suppressor at 1p36, is expressed predominantly from the maternal allele. Further, is there any significance to the observation that in the only two neuroblastoma lines examined with *p73* protein expression, namely IMR-32 and SK-N-SH, this protein was derived from an A/T transcript? At present, we have no experimental evidence for a functional difference between the G/C and A/T alleles. We note, however, that the *p73* transcripts contain an in-frame CUG codon 5' of this polymorphic region, which, at least in several genes, including *basic fibroblast growth factor* and *C-Myc*, acts as an alternative translation initiation codon (Prats et al., 1989). Moreover, conceptual translation from this CUG codon yields a coding sequence somewhat homologous to that of the transcriptional activation domain of p53. We are presently investigating the possibility that this 5' CUG codon is in fact an alternative site for the initiation of *p73* translation.

In this study, we concentrated on neuroblastoma cell lines showing 1p LOH and found that the remaining *p73*

allele lacked mutations similar to those that inactivate p53. However, as only ~30% to 40% of sporadic neuroblastoma tumors display an obvious 1p LOH (Caron et al., 1995), an extensive analysis of neuroblastoma tumors will be required to determine the actual significance of p73 lesions, monoallelic expression, and the A/T polymorphism in the development of neuroblastoma and other diseases.

### p73 Function

The identification of p73, its homology with p53, and its link to tumor suppressors at 1p36 raise fundamental questions regarding p73 function in development and cell cycle control. Does p73, for instance, act in a manner similar to p53 to sense cellular stresses such as DNA damage and hypoxia and integrate this information for cell cycle and cell death regulation? Whereas p73 is shown to be capable of enhancing levels of endogenous p21<sup>waf</sup> protein, it is not induced in cell lines by agents, including UV radiation and actinomycin D, that result in p53 stabilization and activation. Although experiments are under way to determine what signals influence p73 expression, it is apparent that p73 and p53 may be serving distinct functions in the cell. An equally pressing question is whether p73 and p53 interact to yield novel activities not displayed by either molecule alone. Given the dramatic consequences of heterodimerization by members of the c-Myc family (c-Myc, Myb, Max, and Mxi1) on target gene expression (Bernards, 1995), it will be important to determine if such interactions occur amongst p53-like proteins. Our initial efforts focused on the yeast two-hybrid system for testing potential interactions between p53 and p73. Interestingly, both p53 and p73 $\beta$  show strong homotypic interactions, while p73 $\alpha$  has a very low propensity for self interactions in this assay. Moreover, p73 $\beta$  displays an ability to interact with both p73 $\alpha$  and p53, despite its preferential association with other p73 $\beta$  molecules. Although we are presently examining p73-p53 interactions in a variety of cells, the data obtained from the yeast interaction assay suggest that such interactions are possible and may be likely. The analysis of p73-p53 interactions in cells may be especially critical for understanding neuroblastoma, as p53 is often wild type in this disease, and yet recent studies indicate that it is aberrantly cytoplasmic (Moll et al., 1995). An intriguing, though speculative, explanation for this unusual behavior of p53 in neuroblastoma is that p53 requires p73 for some activities that are lost in the absence of p73. Definitive answers to whether p73 has consequential interactions with p53 will obviously require extensive genetic and biochemical studies.

Major questions remain for understanding possible p73 functions in cell cycle regulation and growth control. Curiously, the majority of nonneuroblastoma tumor cell lines was found to express high levels of wild-type p73 transcript and protein, suggesting a role for p73 in proliferation. This observation is perplexing in that most normal tissues show low levels of p73 transcript and protein (data not shown). One possible explanation for elevated p73 in tumor cell lines is that a disruption of normal p53 function, as seen in a majority of these cell lines, results in compensatory or deleterious upregulation of p73 expression. In this scenario, p73 may then either assume

activities of p53 in cell growth regulation or promote survival of a dysregulated cell. Importantly, recent studies have begun to establish links between active cell cycle progression and differentiation (Huttner and Brand, 1997), thereby suggesting a potential mechanism by which p73 might be required for differentiation of neuroectodermal stem cells. Analysis of p73 activity during differentiation and throughout the cell cycle should provide insight into these issues.

It is unclear at present how the identification of this novel gene will impact on our understanding of p53. From an evolutionary standpoint, however, it is interesting to note that p73 shares greater homology with p53-like proteins found in mollusks than with p53 itself. It is formally possible that p53 evolved from a more primitive, p73-like gene involved in many aspects of differentiation and growth control to assume more specific functions in cell cycle control and tumor suppression. It is also possible, given the general quadruplication of the *Hox* complex and other essential genes at the chordate-vertebrate transition (Holland, 1996), that other p53-like genes exist and comprise a p53 regulatory network.

Finally, the discovery of a gene related to p53 will likely contribute to our understanding of the complex etiology of neuroblastoma and other diseases involving 1p LOH and may lead to new avenues in developmental and cell cycle regulation, tumor biology, and alternate strategies for cancer therapy.

### Experimental Procedures

#### Tissue Culture

All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4 g/l glucose, 10% fetal calf serum, 2 mM glutamine, and 5000  $\mu$ g/ml penicillin and streptomycin.

#### Two-Hybrid Assay

The two-hybrid assay was performed essentially as described (Gyuris et al., 1993). Nucleotide sequences corresponding to amino acids 72-393 of p53, 85-636 of p73 $\alpha$ , and 85-499 of p73 $\beta$  were introduced into the vectors pEG202 (resulting in a fusion protein with the LexA DNA-binding domain) and pJG4-5 (resulting in a fusion protein with a transcriptional activation domain). The yeast strain EGY48 was transformed with a pEG202 plasmid, a pJG4-5 plasmid, and the pSH18.34 plasmid containing the *lacZ* gene under the control of eight *lexA* operators.

#### p21<sup>waf</sup> Protein Induction Assay

p73 cDNAs were cloned into a pCMV1 vector containing a 200 bp *lamin* 5'-untranslated region and encoding an N-terminal myc tag (Heald et al., 1993). pCMV-p53 and pCMV-p53V143A were gifts of Dr. Bert Vogelstein (Baker et al., 1990). IMR-32 cells were transfected on 100 mm plates with 20  $\mu$ g of the indicated plasmid and extracts prepared by lysis in SDS sample buffer. Lysates were fractionated on a 10% polyacrylamide gel and transferred to Immobilon membranes by electrophoresis. Membranes were probed with a p21 polyclonal antibody (Calbiochem) and developed by chemiluminescence (Pierce).

#### DNA Damage Response

Cell lines, including IMR-32, MCF-7, and SK-N-SH, were grown to 50% confluence and incubated with either 1 nM or 1  $\mu$ M actinomycin D (Calbiochem) for 24 hrs and 2 hrs, respectively. Cells were then washed with PBS, lysed in 2 $\times$  SDS sample buffer, fractionated on 10% polyacrylamide gels, and electrophoretically transferred to Immobilon (Millipore) membranes. Membranes were probed with a polyclonal antibody to p73 $\alpha$ , a monoclonal antibody to p53 (ATCC,

Bethesda), or a monoclonal antibody to p21 (Calbiochem). For examining responses to ultraviolet radiation, media was removed from plates and cells exposed to 20 and 100 Joules/m<sup>2</sup> 254 nm UV-C. Media was returned to the plates and cells grown for an additional 15 hrs prior to lysis and Western blotting, as above.

#### Fluorescence in Situ Hybridization (FISH)

The p73 cosmid probe was labeled by nick translation using biotin-16-dUTP (Boehringer Mannheim) according to a BRL (Bethesda Research Laboratories) protocol. Twenty microliters of the hybridization solution containing 40 ng of biotinylated probe and 10 µg of human placental DNA was incubated at 80°C for 5 min. DNA was allowed to reanneal at 37°C for 6 hrs before placing on the slides. Chromosome spreads of peripheral blood lymphocytes and the SK-N-AS, SK-N-MC, and IMR-32 neuroblastoma cells were prepared from asynchronous populations grown in DMEM with 10% fetal bovine serum and exposed to colcemid (500 nM) or nocodazole (100 nM) for 2 hr. After hybridization in 50% formamide, 2× SSC, and 10% dextran sulfate for 12 hr at 37°C, biotinylated probes (p73, D125) were detected with an FITC-conjugated anti-biotin antibody (GIBCO), and the digoxigenin-labeled probe (D125) was revealed by a rhodamine-conjugated anti-digoxigenin antibody (Boehringer Mannheim). Chromosomes were counterstained with either DAPI (4,5-diamino-2-phenylindole) or propidium iodide, and slides were mounted in antifade solution (Vectastain).

#### Transfections and Immunofluorescence

Construction of the myc-tagged p73 vectors, transfection, and immunofluorescence were done essentially as described (Heald et al., 1993). Cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS), blocked with 3% milk in PBS containing 0.1% Triton X-100 (PBST), and sequentially incubated with primary and CY3-conjugated secondary antibodies (Jackson ImmunoResearch) for 30 min each at room temperature. DNA was labeled using Hoechst dye 33258 (Sigma) at 1 µg/ml for 1 min. The 9E10 mouse monoclonal antibody against the c-Myc epitope was obtained from the Cell Culture Facility at Harvard University. The rabbit polyclonal anti-p73α antibody was generated against a C-terminal p73α (427–636) glutathione S-transferase fusion protein.

#### SK-N-AS Colony Formation Assay

SK-N-AS cells on 100 mm plates were transfected with the indicated pCDNA3 vectors using the calcium phosphate method. Forty-eight hours later, cell extracts were prepared, immunoblotted, and probed with antibodies to p73, p53, p21, and Bcl-2. Identical sets of plates were grown in 500 µg/ml G418 (GIBCO) for 3 weeks, fixed, stained, and counted (Baker et al., 1990).

#### Molecular Biology Methods

RNA preparation, Northern blot analysis, immunoblotting, genomic and cDNA cloning, as well as screening and nucleotide sequencing, were performed using standard protocols (Maniatis et al., 1992).

#### p73 Gene and Transcript Analysis

cDNA synthesis was performed using 5 µg of total RNA incubated in a 20 ml volume reaction containing 50 mM Tris-HCl [pH 8.3], 10 mM DTT, 10 mM KCl, 0.5 mM dNTP, 30 µg RNasin (Promega), 150 µg superscript II reverse transcriptase (GIBCO; BRL) for 1 hr at 37°C. PCR reactions were performed with either 2 ml of reverse transcriptase products or 300 ng genomic DNA in 50 ml reaction containing 50 mM Tris-HCl [pH 9.2], 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.75 mM MgCl<sub>2</sub>, 10% DMSO, 0.3 mM each primer, and 3.5 µg of TAQ and PWO DNA polymerases (Boehringer Mannheim). The amplification sequence consisted of 30 cycles of 95°C/0.5 min, 58°C/1 min, 68°C/2.5 min after starting with a denaturation step at 95°C for 1 min and ending at 68°C/10 min. The amplicons were purified by spin dialysis sequentially on S400 and P10 resins. For restriction length analysis on transcripts, amplicons were generated as described above using primers (5'-CGGGACGGACGCCGATG and 5'-AGACCGTAGACCGTC ATC derived from the p73 cDNA sequence and analysed on 1.5% agarose gels after digestion with NarI and StyI restriction endonucleases. Restriction length analysis for genomic DNA was performed using two sequential, nested PCR reactions. For PCR-1, primers

5'-CACCTGCTCCAGGGATGC and 5'-AAAATAGAAGCGTCAGTC derived from intronic sequences were employed. For PCR-2, 2 ml of purified PCR-1 reaction products were used with a more internal set of primers (5'-CAGGCCCACTTGCCCTGCC and 5'-CTGTCCCCAAG CTGATGA). The resulting amplicons were StyI digested and analysed on 1.5% agarose gels.

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**EMBL Accession Number**

DNA sequences corresponding to the human p73 $\alpha$  and p73 $\beta$  cDNAs have been deposited in the EMBL database under the accession number Y11416 EMBL.

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